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THE SYNTHESIS OF STARCH FROM CARBON DIOXIDE
USING INSOLUBILIZED STABILIZED ENZYMES

Study Performed by the Lawrence Berkeley Laboratory, Chemical
Biodynamics Division.

Supported by Interagency Contract from National Aeronautics
and Space Administration to the U. S. Atomic Energy Commission.

Order No. A-68042A

James A. Bassham, Principal Investigator
Laboratory of Chemical Biodynamics

Lydia Bearden, Biochemist
Laboratory of Chemical Biodynamics

Charles Wilke, Professor, Chemical
Engineering, University of California

Paul Carroad) Students, Chemical
George Mitra) Engineering, University
Richard Ige) of California, Berkeley

June 9, 1972

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FOREWORD

The energy for life comes from the sun in the form of light. Plants absorb sunlight, and through the energy conversion and synthetic process known as photosynthesis convert water and carbon dioxide to carbohydrates such as starch and to gaseous oxygen. Some men have long dreamed of supplementing this process with practical abiological synthesis of carbohydrates from CO_2 and water. For both technical reasons and economic reasons this has remained a dream.

The time when an economic synthesis of starch in a factory will become a reality is still in the future --- in the range of ten to thirty years. The advent of a technology for using insolubilized, stabilized enzymes makes it possible to consider the synthesis of starch from CO_2 and hydrogen gas with high efficiency. The hope of huge amounts of inexpensive energy becoming available either by atomic fusion or by capture and conversion of solar energy raises the possibility of converting water to oxygen and hydrogen at a sufficiently low cost to rival photosynthesis.

The specter of future widespread starvation arises from the increasing population and the distinct possibility of some form of ecological collapse leading to a sudden catastrophic decline in food production. Such apprehensions provide ample motivation for consideration of systems for artificial manufacture of starch and for delineation of technological areas requiring some years of research before such a system could become practical.

This report first details the rationale for making such a study. Then follows a discussion of the enzyme-catalyzed routes of synthesis available and a choice as to the most promising route. Because so many factors important to this choice are not known accurately, and some technology is not yet

developed, the choice must be tentative. For the guidance of future workers, there is a discussion of enzymes involved, of enzyme insolubilization technology, of possible engineering approaches, with examples in the form of model calculations for both reactors and separators. There is a section on some of the other problem areas. Each section has its own bibliography.

It is hoped that this study will be a useful starting point for other, more thorough studies of systems leading ultimately to the achievement of this important goal. It is also hoped that scientists and engineers will be motivated to seek, and government agencies to grant the support necessary to carry out the research needed to make the dream a reality.

PART I

Introduction and Selection of Synthetic Pathway

James A. Bassham

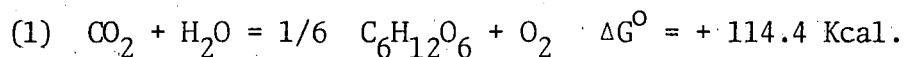
THE SYNTHESIS OF STARCH FROM CARBON DIOXIDE
USING INSOLUBILIZED STABILIZED ENZYMES
An Alternative to Intensive Agriculture?

James A. Bassham

INTRODUCTION

All life requires a continuous supply of chemical energy. For most life on earth, including man, the ultimate source of such energy is the photosynthetic conversion of water, carbon dioxide, and other minerals to oxygen, carbohydrates and other organic compounds. The energy for photosynthesis comes from the sun in the form of the visible light energy absorbed by the plant's pigments: chlorophyll and accessory pigments.

The most abundant immediate organic products of photosynthesis in green plants are the carbohydrates, especially starch, which is made of great numbers of glucose molecules linked together. The equation of the formation of glucose and oxygen from water and carbon dioxide during photosynthesis is given by:



The one sixth mole of glucose plus the mole of oxygen gas together contain 114.4 Kcal. more than the water and CO_2 from which they were formed. This energy came from sunlight. It can be released again when glucose is oxidized by oxygen to make water and carbon dioxide.

Man and other animals, aerobic bacteria, the non-green parts of plants, and even the green tissues of plants in the dark all depend on this release of energy for their life processes. Without the organic products of photosynthesis and gaseous oxygen, most life would cease to exist.

A considerable fraction of the people in the world today are unable to obtain enough food to provide sufficient energy for good health. There is, of course, an additional problem of poor nutrition, particularly protein deficiency. But even on the basis of calories alone, many people are without a sufficient supply of energy to maintain a healthy existence. This problem will become much more severe during the next several decades. This is not a statement of opinion, but a virtually certain prediction, based on present population, its age profile, and the predictable world food supply and distribution facilities. While most students of this problem agree on the need for some decrease in the population growth rate, and some hope for significant progress in this area, even the most optimistically low estimates of population by the year 2000 show a very large increase over the present level.

There have been impressive gains in agricultural productivity in overpopulated countries as modern agricultural practices have been brought to those countries. New hybrid strains of rice and other grains have brought temporary relief from chronic famine in some areas. The application of chemical fertilizers, pesticides, mechanized tilling, planting, and harvesting have all helped to hold off famines which would otherwise have kept the population in check through starvation in India, Bangladesh, and other similar countries.

Can such agricultural miracles continue to prevent disasters greater than those which are already occurring from time to time? After all, many of the famines which we have witnessed recently have been due to wars and other social aberrations. Unquestionably, modern agriculture practices can be considerably extended in underdeveloped countries beyond their present usage.

There are several reasons for pessimism on this score. Some of these reasons involve economic and social institutions, and their inability to deliver the fruits of modern technology where they are perhaps most needed. These problems are beyond the scope of the present discussion, and in any event would affect just as adversely the proposal made in this report. More pertinent to the present discussion is the inherent ecological frailty of increasingly intensive modern agriculture.

As many ecologists have so eloquently noted, there is great danger in massively reducing the diversity of species. When man eliminates most of the plant and animal species over a wide area in order to concentrate on the production of a few crops, there is a greatly increased risk that a major portion of the remaining, widespread species may succumb to the attack of a newly introduced (or long dormant) disease or predator. This is partly because the attacking agent finds an unlimited supply of its energy source; that is, its food. It is partly because its natural enemies are most likely among the species already eliminated or depopulated. And it is because the intensively cultivated crop may be a highly bred new strain in which genetic information necessary to develop resistance has been removed through breeding.

Not long ago, one of these high-yield varieties of maize (corn) which had been widely adopted in the United States became the victim of a plant disease. A substantial part of the crop was lost. Newly developed kinds of rice have greatly increased the productivity of the main food staple in parts of Asia. This increased productivity has made possible further population growth in some already heavily populated areas. Consider the magnitude of the disaster if, at some future date, 80% of the annual crop of rice in these areas should be lost due to the spreading of

some long dormant rice disease for which this high-bred strain had no resistance.

There are other severe ecological problems resulting from the spreading of intensive, widespread "modern" agriculture. Runoff of DDT and other pesticides into rivers, lakes and oceans; runoff of excessive amounts of fertilizers and subsequent eutrophication of lakes and streams; the long-recognized and still serious problem of wind and water erosion of topsoil (and consequent silting of waterways); loss of nutritional value in plants fed nitrate and phosphate but no trace elements... These are a few of the adverse effects of modern agricultural practice.

Recognizing all of these undesirable results of man's present agriculture, one can perhaps be excused for dreaming about the possibility of turning back much of the earth's surface to a multi-specied state. No doubt, one would want to preserve the productivity of fruits and vegetables, perhaps with the gardens and groves interspersed with stands of trees and shrubs, and fields of grass. But the great areas now devoted to intensive cultivation of cereal crops could be perhaps reduced to 25 % of their present extent, with the rest left for parks and natural areas.

Given the present and increasing serious food crises in the world, the reduction of agriculture on the scale just proposed can hardly seem to be more than a dream. And indeed, it is likely to remain a dream through the next two critical decades. There is a possibility, however, that the dream could become a reality in time, even without a substantial population reduction. It could become a reality if we can learn how to synthesize man's principal energy food, starch, economically in factories. By this I do not mean synthesis of starch from other forms of organic matter such as petroleum, for we are rapidly running out of such energy rich-substances.

What is needed is the economic synthesis of starch from carbon dioxide.

There are a number of technological problems to be solved before such an economic synthesis can be achieved, and these will be discussed later. The overriding problem, however, is one of energy. Given a really inexpensive and abundant source of energy, the other problems are all capable of solution within 20 years or less, depending on the level of research effort.

Where could this energy come from? At present the industrialized nations, such as the United States, are facing an energy crisis. Sources of energy such as geothermal power, liquified coal, or even breeder reactors may help meet this crisis but are not likely to provide energy in the quantity and at the low cost required for economic synthetic food. There are, however, two possible sources of massive low-cost, enormous energy supply. Both involve nuclear fusion reactions.

Fusion reactions in the sun are the source of sunlight, which is already the source of energy for nearly all life on earth. There is more than enough sunlight falling on deserts and other nonproductive areas of the earth to provide not only all of our non-biological energy requirements, but also to fulfill our biological requirements as well. There is now a rapidly growing interest in solar energy conversion. It is a reasonable supposition that the development of solar energy as an economic source of power will be the next big national technological commitment in the United States.

Naturally, at the present time, solar energy conversion looks uneconomic. Even its protagonists talk about solar energy becoming more economically attractive after not only the technical problems have been solved, but also when competing energy sources have become much more expensive (as

seems certain).

While this pessimism is probably warranted for the next decade or so, it may be unrealistic for the future - say beyond the year 2000. Much of the present pessimism is based on the high cost of solar cells built of silica crystals for the space program. Electrical energy produced by such devices costs about 1000 times too much to be competitive with present energy sources.

Solar energy falling on the desert is free. All we have to do is to convert it to electrical or chemical energy with some mass-produced, thin-layered device capable of efficiency somewhere comparable to that of the green cells of plants - that is about 40 %. We would do well to be guided in our design by the mechanism used in the photosynthetic cell. Unfortunately, we don't know these mechanisms in all their details. We do know quite a lot and are learning more rapidly.

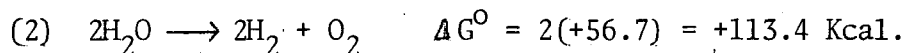
The photosynthetic energy-converting apparatus consists of a number of very thin membranes, with pairs joined at the edges to make very thin discs, called thylakoids. The spaces inside these discs are all interconnected by a fretwork of flat tubules. The membranes themselves appear to be largely nonconducting (insulators) but contain protein-lipid complexes imbedded in the phospholipid bilayers. These complexes are the points of initial conversion of electromagnetic energy to electrochemical energy.

Light energy is first absorbed in the chlorophyll and other pigment molecules, and the absorbed energy in the form of an excited state of the electrons in the pigment molecules can then jump from one pigment molecule to another. Perhaps more correctly, we can think of the whole array of pigment molecules within a certain distance as sharing the excitation

energy. This energy packet, or "exciton" is transmitted in a very short time to the energy conversion complex. There the exciton causes an electron to be promoted out of a pigment molecule and onto some acceptor, according to present theories. The oxidized pigment molecule (the one that has lost an electron) will eventually recover it from water. After two molecules of water have lost four electrons this way, a molecule of gaseous oxygen, O_2 , evolves.

In the meantime, the electron promoted out of the chlorophyll molecule is being transmitted from one carrier to another, and will eventually undergo another photochemical "promotion" through a second energy conversion center which has received another exciton. By this time, the electron is at the chemical potential of gaseous hydrogen, and H_2 could be evolved by the lamellar system. This does not happen ordinarily, as the plant has other uses for this reducing power. Instead, the electrons at the potential of H_2 are used to reduce carbon dioxide to carbohydrates, including starch.

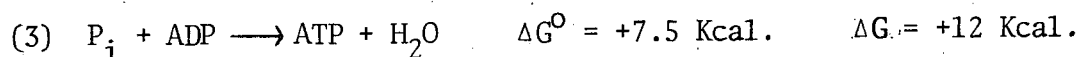
The energy used in this process, two light photons of wave lengths shorter than 700 nm, is considerably more than would be required to take an electron from water and raise it to the potential of hydrogen gas. The energy per mole of photons (per einstein) for 700 nm light is 40.5 Kcal, so that two einsteins is worth 81 Kcal. The energy to split two molecules of water to oxygen and hydrogen is $2 \times 56.7 = 113.4$ Kcal.



Since four electrons must be transferred in this reaction, the energy per mole of electrons (per equivalent) is $113.4/4 = 28.4$ Kcal. Thus the two einstein to one equivalent process is $28.4/81 = 35\%$ efficient.

However, in the process of moving the electron up to the potential of

H₂, the photochemical system also manages to use some of the left over energy to convert the biochemical acids, phosphate and adenosine diphosphate (ADP) to their anhydride, adenosine triphosphate (ATP) and water, thereby conserving another 12 Kcal.



Here, $\Delta G = 12 \text{ Kcal.}$ is greater than ΔG^0 because of the low concentration of P_i and the high ratio of ATP concentration to ADP concentration. At least one ATP molecule is made for each two electrons transported, so the overall efficiency in the lamella may be calculated to be $(28.4 + 6)/81 = 42 \%$.

There are a number of theories about the physical and chemical mechanisms involved in this efficient energy-conversion process. Great progress towards full understanding has been made during the last twenty years. It seems not overly optimistic to expect that the next decade will bring sufficiently detailed knowledge about the mechanism to permit the principles to be modeled in artificial systems for direct conversion of solar energy to either electrical energy or chemical energy in a usable form. Indeed, some scientists have already built models, based on their current concepts of the photosynthetic mechanisms. These models, while generating electrical energy from light with extremely poor efficiency, nevertheless offer sufficient promise from a theoretical standpoint to warrant extended fundamental investigation.

Aside from solar energy conversion by copies of photosynthetic systems, other attractive schemes utilizing more conventional approaches have been proposed and are being studied. If any such systems become economically feasible and are built, one can predict that energy generation will become less expensive in real dollars as time passes. This prediction is

based on the fact that the system is based on a free and inexhaustible energy source.

The second type of energy from atomic fusion reactions is fusion power plants on earth. There have been great hopes for inexpensive power from fusion reactors in the past. Unfortunately, progress towards a workable system has not been as rapid as hoped, even though significant gains have been made in the length of time that the ionized gas (plasma) can be contained in its magnetic "bottle". Our present limited commitment to fusion reactors in the United States may not be sufficient to produce fusion power before the year 2000. There is, however, a distinct possibility that the U.S.S.R. may achieve important new breakthroughs during the next decade, and that might stimulate the U.S. to make a greater effort also. Thus, there is a reasonable possibility that abundant, inexpensive nuclear power might become available in 20 years.

Possible Interim Need for Artificial Starch Synthesis

The foregoing section suggests, in consideration of the long range future-say beyond 1995, there is a rational argument for beginning now to develop the techniques of synthesis that would permit the synthesis of carbohydrates from CO_2 under the expectation that by that time sufficient quantities of energy may be available at a low enough cost to make such a synthesis attractive. What about the less long-range future, the period beginning about 1980? Is it conceivable that by that time population and nutritional circumstances in parts of the world might be so desperate as to warrant heroic but somewhat uneconomic measures solely for the preservation of life? This is quite a different presumption from the earlier argument where it was assumed that setting up factories for the synthesis of starch might become an attractive option to compete with total reliance

on conventional agriculture.

Certainly the population in presently underdeveloped and overpopulated countries is going to increase alarmingly by 1980, even if birth-control measures now being attempted are moderately successful. A significant falling off of a major cereal crop such as rice due to any one of a variety of adverse events (disease, abnormal weather, war and social disruption, etc.) could easily trigger a condition of famine which might persist for several years. The "excess" production by North American granaries has been able to supply enough cereal crops to alleviate famines in the past, but these surpluses are likely to grow somewhat smaller as North American population grows and other foreign markets increase due to population growth in other countries with large populations and marketable goods for trade. Also there is the severe problem of overseas shipment and distribution when a demand suddenly develops.

Given such circumstances, a starch factory located in the country of need might well be allocated power even if uneconomical. In the choice between calories of food for survival, and other energy requirements, a government might well allocate its power for food.

Of course, such a sophisticated and expensive factory as will be required is not going to be built in time during an emergency. It might, however, be built ahead of time as a pilot project, by cooperation between the local government and contributions from the developed countries. Possibly it could be part of a complex which would also include power generation by atomic fission, perhaps a new generation breeder reactor. The plan would be that the power plant could mainly allocate its power for other purposes when there was no serious food shortage. The starch factory could be run on a limited basis, at times of day when there is less

energy demand. Then, if famine struck, the starch plant could be operated at full capacity and could temporarily take the entire power output of the power plant. Thus the starch factory would be justified on the basis of insurance against disaster and the development of experience in food synthesis rather than on the basis of the marketability of its product in normal times.

Problem Areas in Building a Starch Factory

In the foregoing section I have speculated on the possibility that governments might want to build a starch factory as soon as 1980. This sounds rather optimistic in terms of social institutions being intelligent enough to plan for disaster before it strikes. There is perhaps an even greater optimism in thinking that the technical problems involved in the construction of such a plant could be solved by 1980. As we shall see, these problems are formidable, and only the application of a substantial research effort beginning in the near future would bring any possibility of solutions by such an early date.

The need for such factories will grow inexorably in the future. It seems safe to predict that eventually the necessary research will be undertaken. Many research aspects are already being undertaken in connection with more immediately attractive projects. Thus there is rapidly growing literature on the use of insolubilized enzymes in industrial processes. Research on dialysis and osmolysis membranes, presently being used for such diverse problems as medical research and water purification, may provide materials helpful in carrying out the severe separation problems described later.

Other problems more specific to the synthesis of starch will require extensive research. Preeminent among these are the insolubilization and

stabilization of enzymes catalyzing the synthetic reactions of starch synthesis from CO_2 . Also enzymes needed to regenerate ATP from ADP and inorganic phosphate and to reduce biological electron carriers such as ferredoxin and nicotinic adenine dinucleotide phosphate (NADP) must be studied in this way. Most work on stabilized enzymes to date appears to have been focussed on enzymes required to break down polymeric molecules such as proteins into their subunits. Certainly most of the industrial applications have been limited to such hydrolytic reactions. Unfortunately, many of the enzymes which would be involved in synthetic paths are more complex and of higher molecular weight (for example the enzyme ribulose diphosphate carboxylase) and may prove more difficult to stabilize.

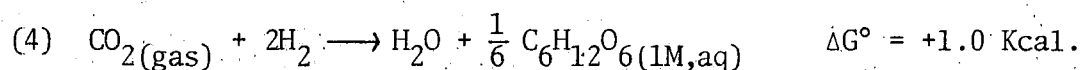
Aside from the enzyme problem, any conceivable synthetic process leading from CO_2 to starch will require a number of difficult separations of metabolites and other substances in the output from the reactors. Even assuming rapid and significant advances in selective membrane technology these problems loom as very difficult and possibly limiting to the entire project. Even though present technology for reactors with insolubilized enzymes may favor carrying out one or two biochemical steps at a time, economy of separations may dictate a multicomponent reactor. Thus there is a tradeoff which must be solved by systems engineering.

Among other severe problems may be mentioned the collection of carbon dioxide from the atmosphere where it occurs at a partial pressure of only 0.03 % (300 ppm). These and other problems will be discussed in more detail later.

SELECTION OF SYNTHETIC PATHWAYS

Chemical Energetics

Before proceeding to a discussion of the optimal synthetic path for the synthesis of starch from CO_2 , it is useful to consider the overall energetics of this process. From Equations 1 and 2 (Introduction), it is clear that the energy for the formation of oxygen and of one-sixth glucose molecule from carbon dioxide (gas) and water (+114.4 Kcal) is virtually the same as the energy for the formation of two moles of hydrogen gas and one mole of oxygen gas from two moles of water (+113.4 Kcal). If Equation 2 for the splitting of water to hydrogen and oxygen is subtracted from the photosynthetic equation (Equation 1) we see that the reduction of carbon dioxide to water and glucose (Equation 4) is essentially an equilibrium process, with very little free energy change. (Free energy changes in this and other equations in this section are mostly based on a table of free energy data by K. Burton, which appeared as an appendix to an article by Krebs and Kornberg in 1957).

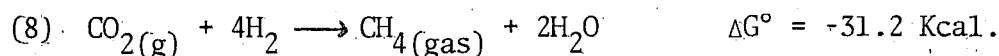
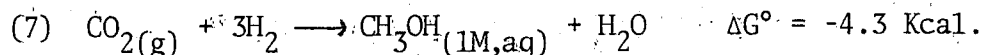
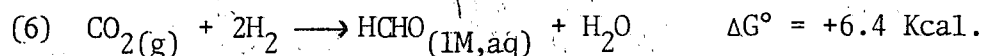
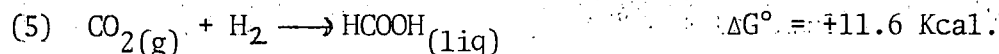


Equation 4 tells us that for the reduction of CO_2 to glucose, hydrogen gas is nearly sufficient. If the reaction were a simple one (few steps), it might even be possible to force it, without other inputs of chemical energy, by simply increasing the pressure of H_2 and CO_2 . However, the reaction pathway is quite complex, involving many steps, and additional input of chemical energy is required to make the reactions proceed at a practical rate. Also, the desired end product is not free glucose, but starch, and additional chemical energy is required to link up glucose residues by removing water. Thus we shall see that green plants use not only

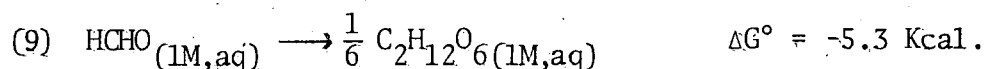
the equivalent of two moles of H_2 gas per mole of CO_2 reduced to starch, but also $3 \frac{1}{6}$ moles of ATP, the biochemical molecule most commonly employed to provide additional chemical energy.

In our consideration of synthetic paths for the reduction of CO_2 to starch we start with the assumption that the supplied electrical energy from the solar energy converter or atomic reactor will be used to electrolyze water to make hydrogen and oxygen. This will be the major energy input, at least for the chemical process. Of course, hydrogen could be obtained with less energy cost from other sources, such as hydrocarbons, but it is a basic assumption of this study that only completely oxidized raw materials will be used. If by the time starch synthesis from CO_2 becomes feasible there are still enough petrochemicals to permit their use for food, we assume they will be converted to protein via bacterial metabolism, as is being done even now on a limited scale.

Carbon dioxide can be reduced with hydrogen to four different oxidation levels and these will be of some interest in our discussion of pathways to starch. The reductions of CO_2 to formic acid, formaldehyde, methanol and methane are shown in Equations 5 through 8, respectively:



Formaldehyde is at the same oxidation level of carbon as glucose, and is somewhat less stable:



Reduction of Carbon Dioxide to Formaldehyde

From the foregoing energy considerations, and for the important objective of choosing the simplest possible pathway, one might conclude that the chemical reduction of CO_2 to formaldehyde followed by the enzymatic conversion of formaldehyde to glucose and then to starch should be the method of choice. Despite serious problems with this pathway, described below, this remains an attractive alternative to the pathway which we later recommend.

The first and most serious difficulty is that there appears to be no known way to reduce CO_2 directly to formaldehyde in good yield. This must be due in part to the fact that formaldehyde is unstable with respect to its further reduction to methanol (see Equations 6 and 7). However, it seems possible that further research on this reduction could turn up a catalyst and other reaction conditions which might allow the efficient formation of formaldehyde from CO_2 and H_2 .

Of course the reduction can be allowed to proceed to methanol, and then the methanol can be reoxidized to formaldehyde. Apparently this can be done in satisfactory yield. The objection to such a route is that it uses up an extra mole of hydrogen gas, thus increasing the energy budget for the electrolysis of water by 50%. At the same time, there appears to be no way to recover the energy released in the reoxidation of methanol to formaldehyde.

While a 50% increase in the energy requirement for the electrolysis of water seems a high price to pay, it might be justified if the subsequent enzyme-catalyzed pathway were to be so simplified and the number of steps so reduced as to save a comparable amount of energy in the operation of the enzyme reactors and separation processes. From our analysis we doubt that

there is enough compensating simplicity for this pathway. However, we offer it in more detail later as an alternative to the recommended pathway. In considering this alternative pathway, it will be seen that a second problem is the lack of knowledge about the key enzyme which would be required to bring about the addition of formaldehyde to a five-carbon sugar phosphate. Such a reaction is needed to permit the construction of a cyclic pathway leading to glucose formation and regeneration of the three-carbon acceptor.

Reduction of Carbon Dioxide to Formic Acid

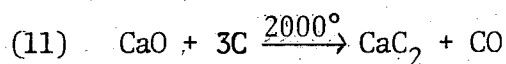
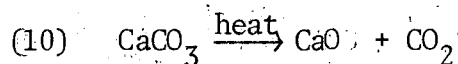
Another possible chemical reduction of CO_2 would be its reduction to formic acid. Further enzymatic reduction would then be required, either of the formate itself, or of some product formed from formate. A survey of enzymically-catalyzed reactions which incorporate formate into larger molecules did not reveal any in which the product could be converted to the carbohydrate level by a few reactions as are required for the conversion of the product of the carboxylation reaction of photosynthesis, discussed later.

Conversion of Carbon Dioxide to Two- or Three-Carbon Compounds by Organic Syntheses

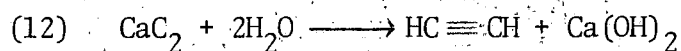
In theory, carbon dioxide could be converted to simple two carbon or three carbon compounds by well known organic reactions. These compounds could then serve as substrates for enzymically-catalyzed reactions leading to carbohydrates. However, the energy cost of these reactions must be kept in mind. Also it is necessary to produce organic compounds that are not stereoisomers, only one of which can be used as a substrate for subsequent enzymically-catalyzed reactions. Finally, the use of non-enzymic steps should result in a shortened enzymic pathway to carbohydrates. We

have not found any routes that would satisfy these three criteria, when compared with the path chosen.

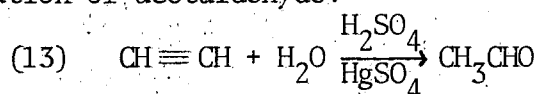
As an example of these difficulties, consider the synthesis of acetaldehyde, via acetylene. Calcium carbonate is converted to CO_2 and CaO by heating (which requires energy), and the CO_2 can be stored for a subsequent carboxylation reaction. The CaO is reacted with C (from coke?) at 2000° to make calcium carbide and carbon monoxide.



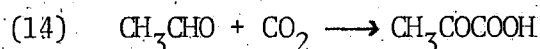
Possibly the carbon monoxide could be burned to make more CO_2 and recover some of the energy as heat. The calcium carbide could then be reacted with water to give acetylene:



Acetylene is a good starting point for a number of syntheses including the formation of acetaldehyde:



Acetaldehyde could be carboxylated by an enzyme from certain bacteria to give pyruvic acid. Unfortunately, even this three carbon compound requires



a number of biochemical steps before it can be transformed to 3-phosphoglyceric acid, which is needed for reduction to a simple three-carbon sugar. These steps are described later, under carboxylation reactions.

A basic premise for this study is that the path chosen should require no raw materials except carbon dioxide, water and electrical energy. Thus a pathway which calls for elemental carbon is unacceptable, unless it also

can be generated from CO_2 . To do so would require an additional large input of energy. Of course, acetaldehyde is a partly reduced compound of carbon, and only one-third as much hydrogen would be needed to convert pyruvate and CO_2 to glucose as is required for converting only CO_2 to glucose. However, the use of heat to split oxygen from CO_2 is likely to consume far more energy than the splitting of water by electrolysis.

As another example of the energetic cost of carrying out the usual pathways of organic chemistry, consider the fixation of CO_2 by a Grignard reaction. All of the ether usually used would have to be recycled, and the magnesium hydroxide produced would have to be converted back to metallic magnesium. But even more difficult, the alkyl halide, for example methyl iodide, would have to be synthesized from CO_2 , H_2 , and iodide. Since many organic reactions are less quantitative than enzymically-catalyzed reactions, such complex regenerative pathways are in most cases likely to be much less efficient from an energy standpoint than pathways catalyzed almost entirely by enzymes. Thus, unless the non-enzymic path were significantly shorter, it could not compete. We have found no such pathway that is shorter -- in fact all appear to be longer.

Enzymically-Catalyzed Reactions, Beginning with CO_2

Having seen the difficulties inherent in utilizing various non-enzymically-catalyzed reactions, including the reduction of carbon dioxide, we turn now to the only other alternative, the carboxylation reactions and subsequent steps catalyzed by enzymes and leading from CO_2 to starch.

The Photosynthetic Carbon Reduction Pathway

The most important pathway for conversion of CO_2 to glucose in nature is the photosynthetic carbon reduction cycle (Bassham, et al., 1954), called the reductive pentose phosphate cycle (Figure 1). This pathway begins with

Figure 1. Reductive and Oxidative Pentose Phosphate Cycles.

Heavy lines indicate reactions of the Reductive Pentose Phosphate Cycle.

Light lines indicate reactions of the Oxidative Pentose Phosphate Cycle.

Enzyme Key

Reductive Pentose Phosphate Cycle (Photosynthesis) only

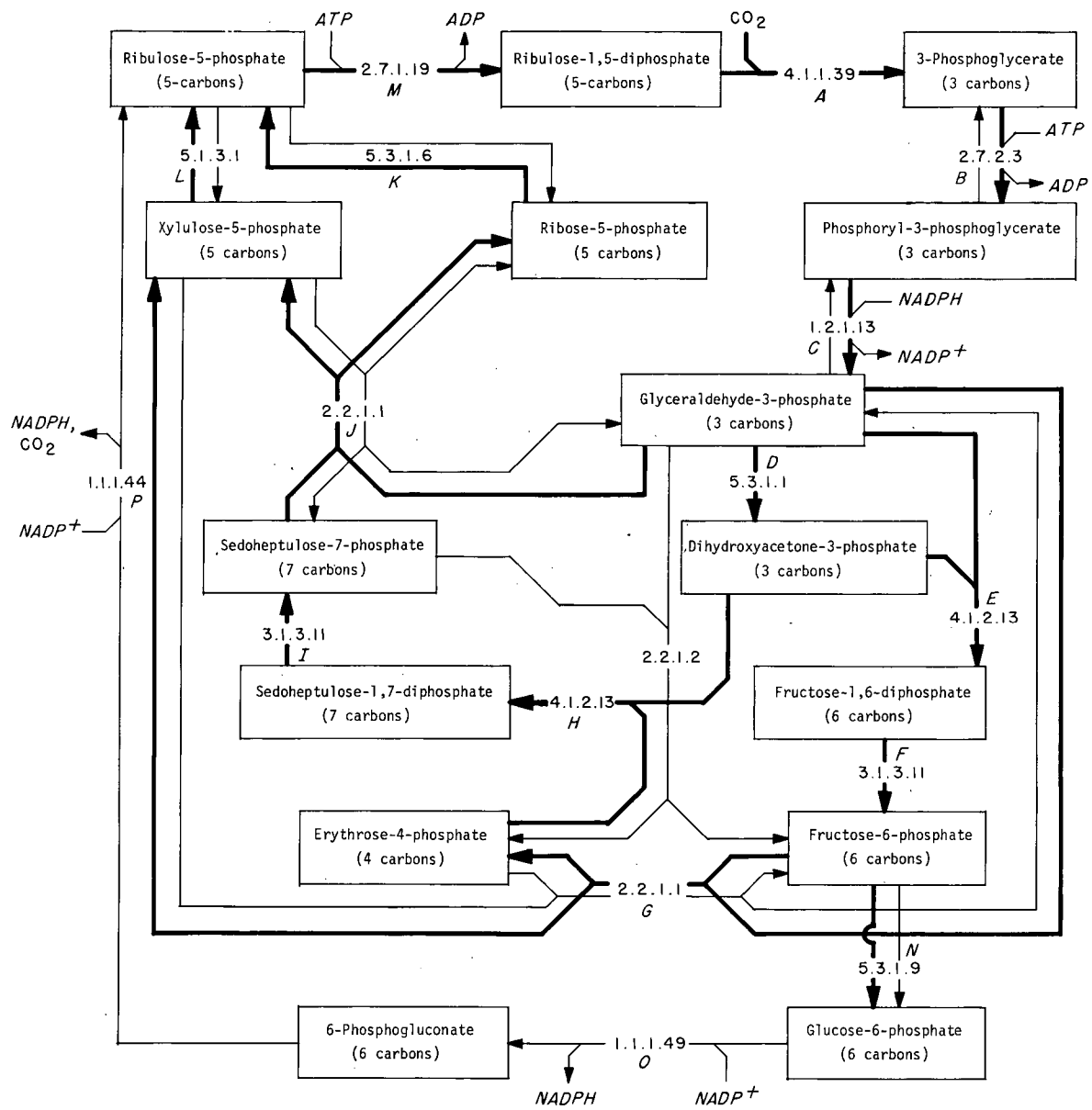
- 2.7.1.19 phosphoribulokinase
- 3.1.3.11 hexose (heptose) diphosphate
- 4.1.1.39 ribulose diphosphate carboxylase
- 4.1.2.13 fructose diphosphate aldolase

Reductive and Oxidative Pentose Phosphate Cycle and Triose Phosphate Oxidation

- 1.2.1.13 triose phosphate dehydrogenase (NADP^+)
- 2.2.1.1 transketolase
- 2.7.2.3 phosphoglycerate kinase
- 5.1.3.1 ribulose phosphate 3-epimerase
- 5.3.1.1 triose phosphate isomerase
- 5.3.1.6 ribose phosphate isomerase

Oxidative Pentose Phosphate Cycle only

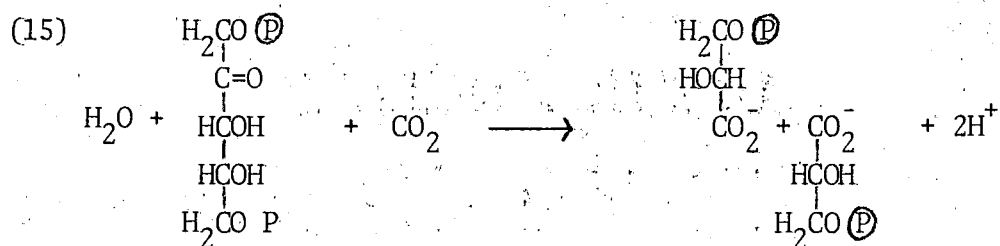
- 1.1.1.44 phosphogluconate dehydrogenase (decarboxylating)
- 1.1.1.49 glucose-6-phosphate dehydrogenase
- 2.2.1.2 transaldolase



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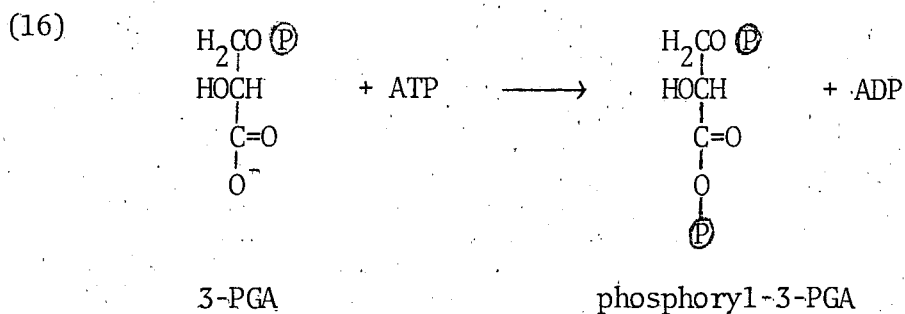
the carboxylation of a five carbon sugar diphosphate, ribulose-1,5-diphosphate. No intermediate six carbon compound is detected, and the products of the reaction are two molecules of 3-phosphoglyceric acid. Since this and other acids are ionized at physiological pH, this and other acids will be referred to in their ionized forms. Also the symbol (P) will be used to denote a phosphate group, $-\text{PO}_3\text{H}^-$. The enzyme catalyzing this carboxyla-



ribulose-1,5-diphosphate D-3-phosphoglycerate

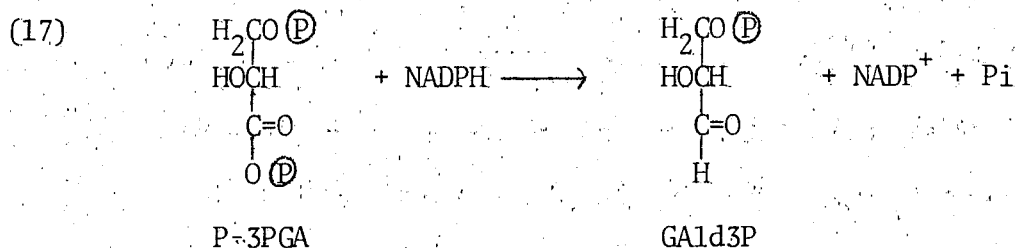
tion reaction is commonly called ribulose-1,5-diphosphate carboxylase.

The sugar acid, 3-phosphoglycerate (3-PGA) is more oxidized than a sugar, and can become a three carbon sugar only after the carboxyl group is reduced to an aldehyde. Since the carboxylate ion is stable (resistant) towards reduction, it must first be activated. The organic chemist might convert it to an acyl chloride, but living cells use the terminal phosphate group of ATP to convert to another kind of acid anhydride, an acyl phosphate. The enzyme for this reaction is 3-phosphoglycerate kinase.



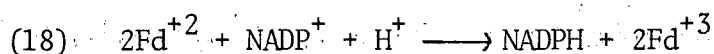
The acyl phosphate may now be reduced. The reducing agent is nicotine adenine dinucleotide phosphate in its reduced form (NADPH). The enzyme is

triose phosphate dehydrogenase.

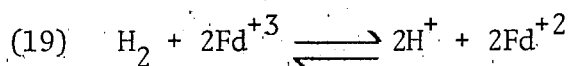


The product of this reaction is 3-phosphoglyceraldehyde (GAld3P), a three carbon sugar phosphate (triose phosphate).

The biochemical reducing agent, NADPH, carries two electrons and, in effect, transfers a hydride ion to the acyl phosphate. The redox potential for NADPH at pH 7 is -0.324 volts, about 0.1 volts less negative than that of hydrogen gas in equilibrium with protons at pH 7, or the reduced form of the non-heme iron protein ferredoxin, both of which have a redox potential at pH 7 of -0.42 volts. Thus NADP^+ is readily reduced in presence of an oxidoreductase by reduced ferredoxin from the light reactions of photosynthesis:



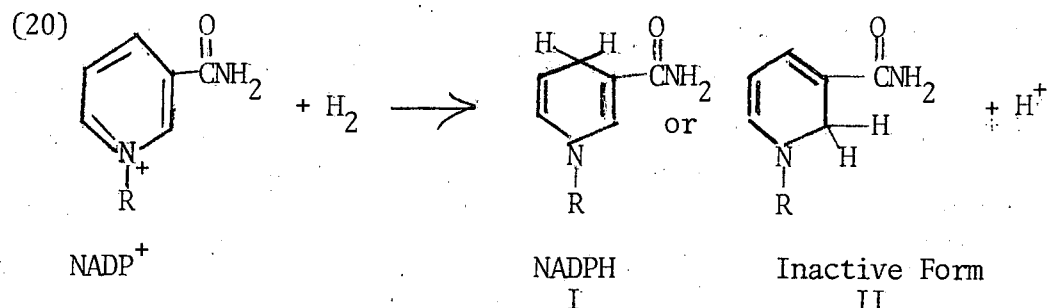
In non-photosynthetic bacteria which utilize hydrogen gas as an energy source, there is a hydrogenase enzyme which can catalyze the reaction between hydrogen gas and ferredoxin:



This is a reversible reaction, and depending on the potential of the particular ferredoxin employed, several atmospheres of hydrogen gas may be required to drive the reaction in the forward direction shown. This direction is also favored by increasing the pH.

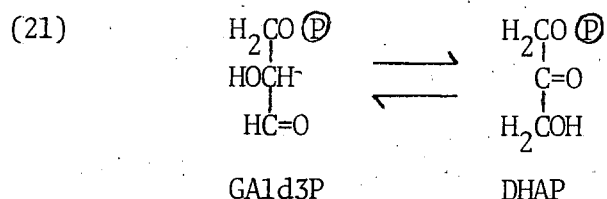
Reactions 15, 16 and 17 provide a direct route for the utilization of the reducing power of H_2 for the reduction of the carboxylation product to

sugar. There may be alternatives to this route such as the electrochemical reduction of either NADP^+ or ferredoxin, or the chemical reduction of either with H_2 and a suitable catalyst. The chemical reduction of NADP^+ could be complicated by the possibility of more than one site of reduction

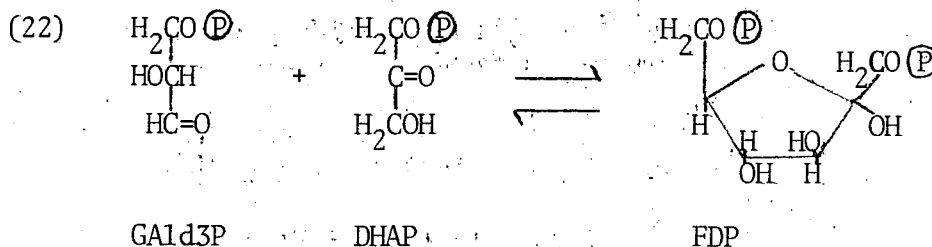


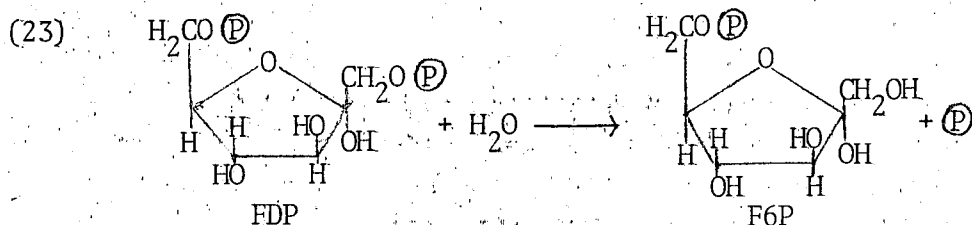
on the pyridine ring of NADP^+ . Form I of NADPH is the one required for the triose phosphate dehydrogenase which is NADP-specific.

Once glyceraldehyde-3-phosphate is formed, no further inputs of chemical energy are required to make glucose-6-phosphate (G6P), the precursor of starch. GAlD3P isomerizes to give dihydroxyacetone phosphate (DHAP). The enzyme for this reaction is triose phosphate isomerase. Then the two triose phosphates undergo an aldol-type condensation catalyzed by aldolase.

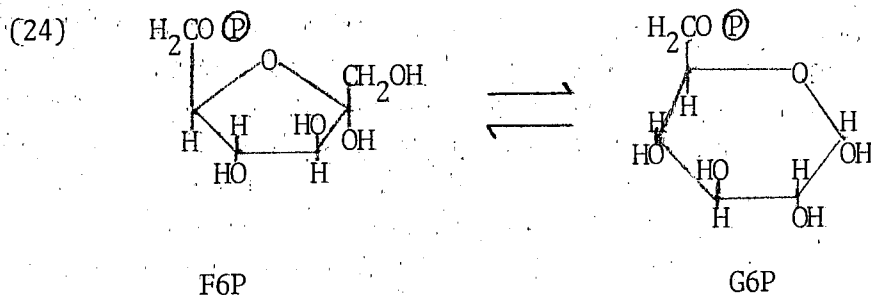


This gives fructose-1,6-diphosphate (FDP) which is half hydrolyzed to fructose-6-phosphate (F6P) in the presence of fructose diphosphatase.

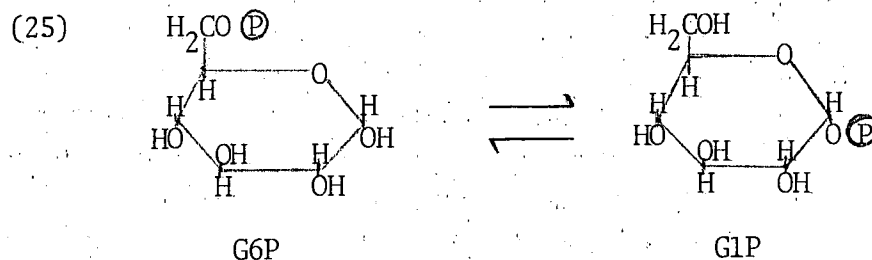




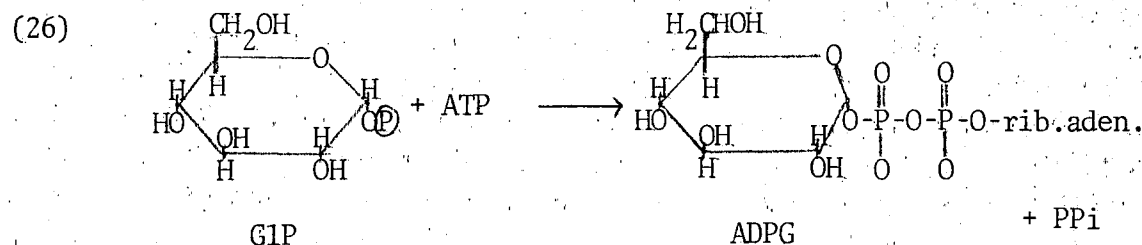
There remains only an isomerization, catalyzed by hexose phosphate isomerase, to convert F6P to G6P.



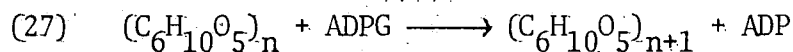
In green plant cells, the conversion of G6P to starch begins with the movement of the phosphate group to the number one carbon position, catalyzed by phosphoglucomutase.



Glucose-1-phosphate then reacts with ATP to give adenosine diphosphoglucose (ADPG) and pyrophosphate (PP_i). The enzyme is ADPGlucose pyrophosphorylase.



This ADPG then reacts with the starch chain to add one glucose residue to the chain. Thus the synthesis of starch from glucose-6-phosphate, as it



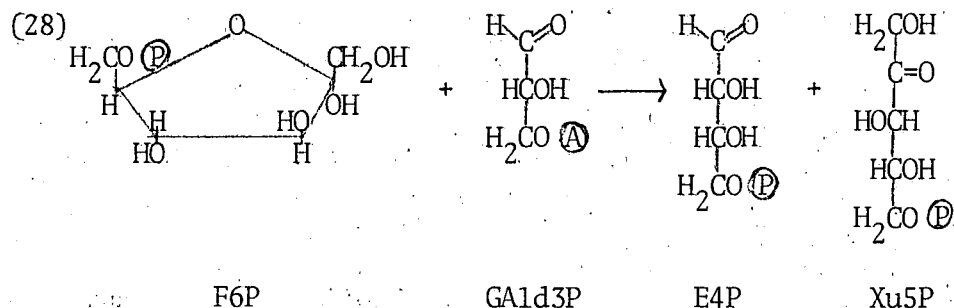
occurs in plants, requires the net conversion of one molecule of ATP to ADP for each glucose residue ($C_6H_{10}O_5$) added to the starch chain.

Completion of the Reductive Pentose Phosphate Cycle

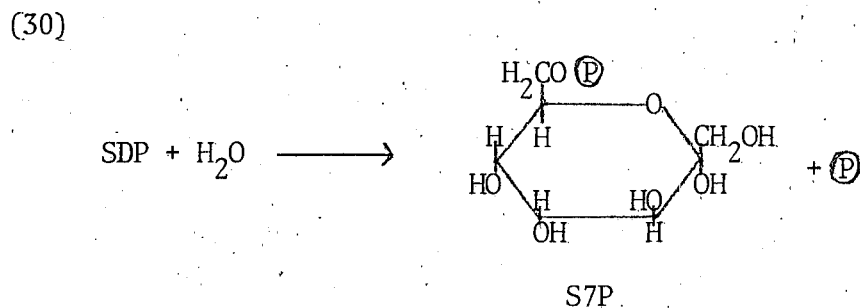
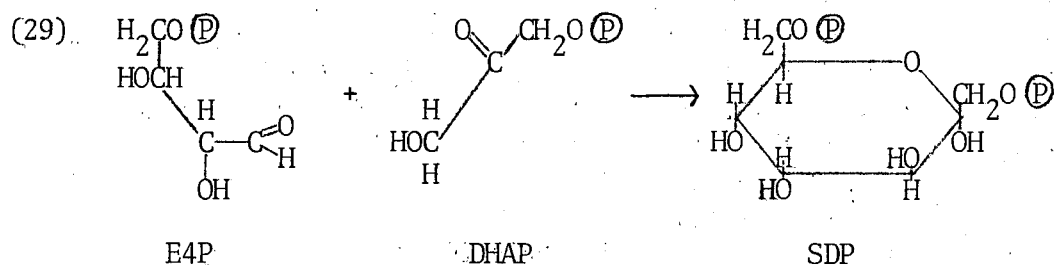
So far we have traced the path of carbon from the carboxylation to starch. However, for the cycle to run, the carbon dioxide acceptor, ribulose-1,5-diphosphate (RuDP) must be regenerated. Clearly, for the compounds of the cycle to be maintained at a constant level, six molecules of CO_2 must be incorporated for each glucose-6-phosphate molecule converted to starch. Each CO_2 molecule taken up reacts with five carbon atoms of sugar (Equation 15), producing two molecules of 3-PGA which are reduced to two triose phosphate molecules. Thus, six carboxylation reactions produce twelve triose phosphate molecules, of which two are used for glucose phosphate synthesis and ten are required to regenerate the six molecules of RuDP. The following process which converts five triose phosphate molecules (15 carbon atoms) to three RuDP molecules (15 carbon atoms) thus occurs twice for each net G6P synthesized.

The first steps in this process are the conversion of two triose phosphates to F6P, and have already been described by reactions 21, 22 and 23. The next step is a reaction between F6P and GAlD3P to give a five carbon sugar, xylulose-5-phosphate (Xu5P) and a four carbon sugar, erythrose-4-phosphate (E4P). This reaction is mediated by the enzyme transketolase. The reaction actually involves the transfer of the carbon atoms one and two of the fructose to the coenzyme, thiamine pyrophosphate (TPP), forming

a glycolaldehyde-TPP compound which remains bound to the enzyme. The remaining four carbon atoms of F6P are released as the aldose phosphate, E4P. The glycolaldehyde-TPP complex reacts with another aldose phosphate, in this case GAlD3P, forming Xu5P.

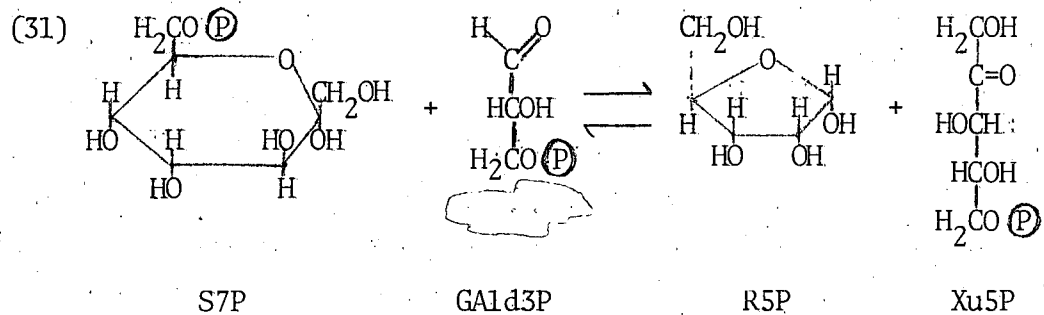


Erythrose-4-phosphate reacts with DHAP in a reaction mediated by aldolase which is analogous to reaction 22, but which produces in this case a seven carbon sugar diphosphate, sedoheptulose-1,7-diphosphate (SDP). This diphosphate also is hydrolyzed to its monophosphate, sedoheptulose-7-phosphate (S7P).

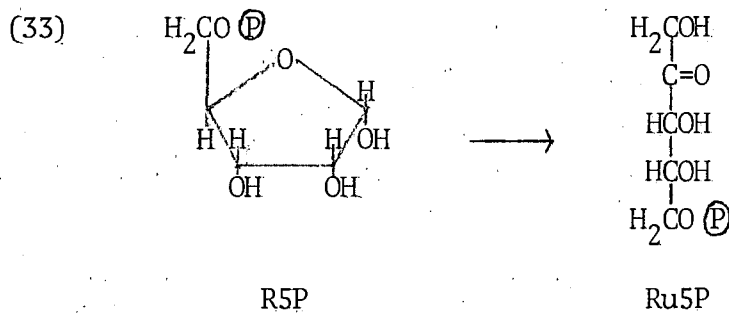
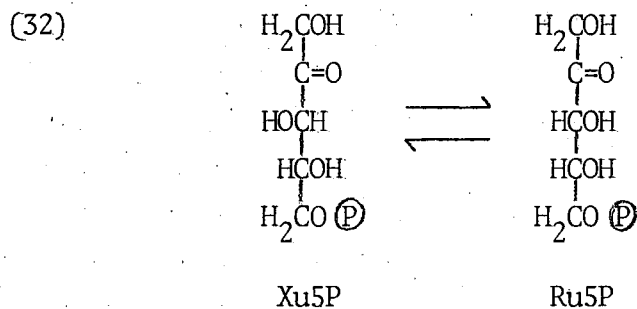


Sedoheptulose-7-phosphate undergoes a transketolase-mediated reaction, similar to Reaction 28, giving another molecule of Xu4P and an aldopentose

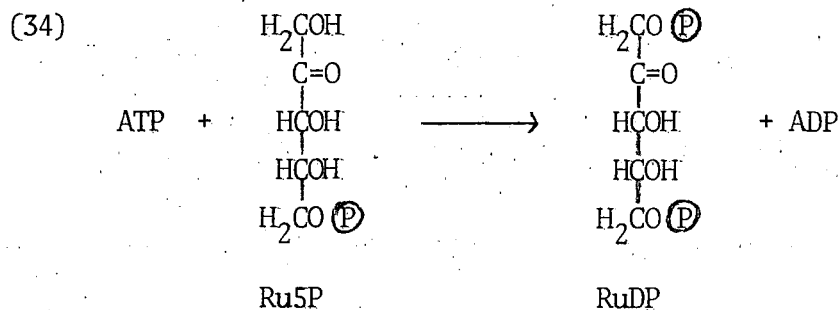
phosphate, ribose-5-phosphate (R5P).



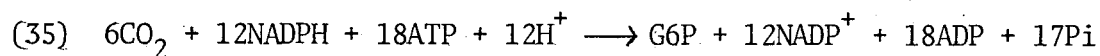
Ribose-5-phosphate is converted to the ketopentose phosphate, ribulose-5-phosphate, by the enzyme ribose phosphate isomerase. Xylulose-5-phosphate is converted to ribulose-5-phosphate (Ru5P) by the enzyme phosphoketopentose epimerase.



This completes the rearrangement of five triose phosphate molecules to three pentose phosphate molecules, but one further step is required to form ribulose-1,5-diphosphate, the carboxylation substrate. This is the conversion of Ru5P to RuDP with a molecule of ATP, mediated by phosphoribulokinase.



This reaction completes the reductive pentose phosphate cycle. The complete net equation for the synthesis of one molecule of G6P may now be written:

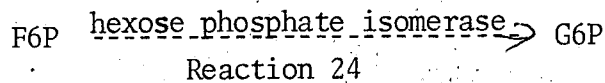
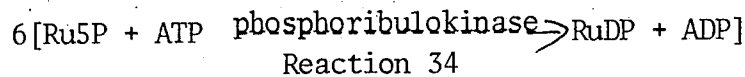
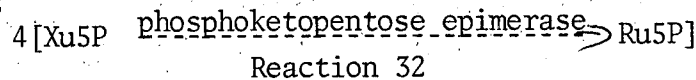
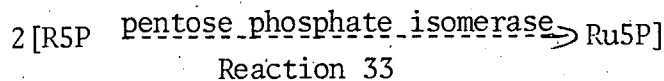
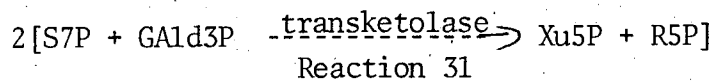
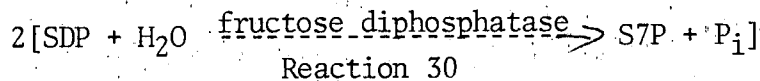
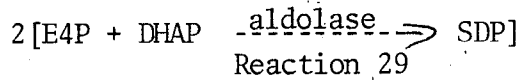
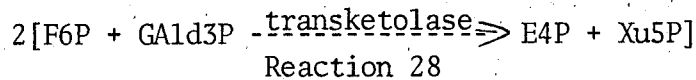
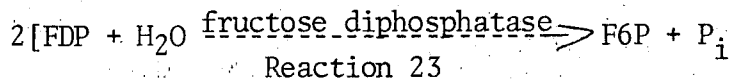
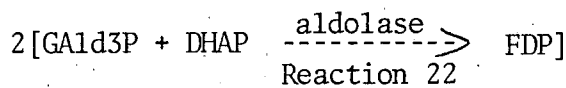
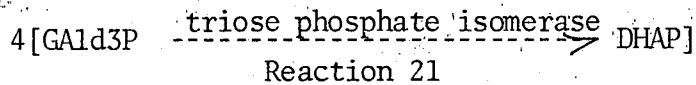
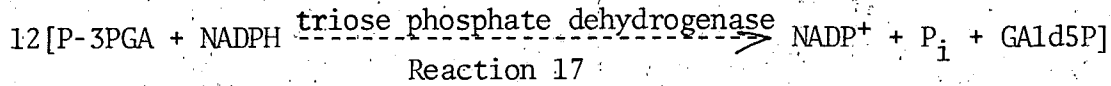
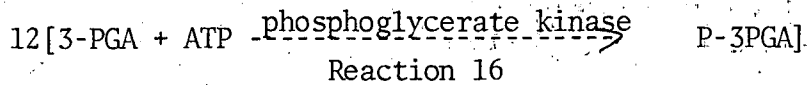
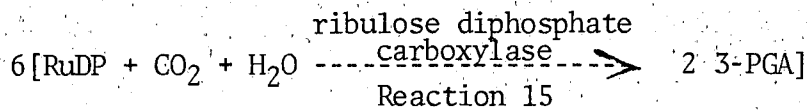


If we add the ATP required to convert G6P to starch, the total requirement comes to 12 molecules of NADPH (coming from 12 molecules of H_2 or its equivalent) and 19 molecules of ATP. For each CO_2 molecule converted to starch, this comes to two H_2 molecule and $3\frac{1}{6}$ molecules of ATP. In our investigations of other possible pathways, none have been found that required a smaller energy input. Since H_2 must come from the splitting of water (Equation 2), each mole of H_2 represents an expenditure of at least 56.7 Kcal. Each mole of ATP formed from ADP and Pi (Equation 3) costs at least 12 Kcal. Actually the cost will be much more, perhaps 25 Kcal per mole, considering the problems of a regenerative pathway, discussed later.

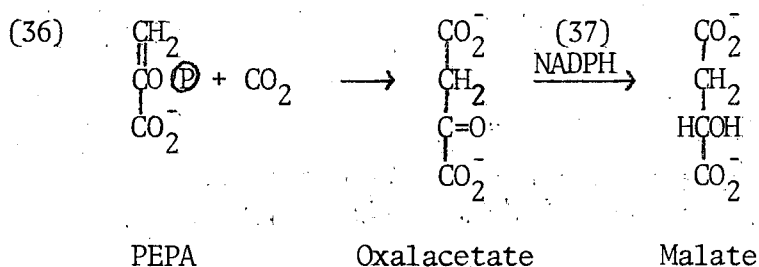
The Pyruvate-Malate Photosynthetic Pathway

For about 10 years, the reductive pentose phosphate pathway was thought to be the only photosynthetic pathway for the incorporation and reduction of carbon dioxide. Then a second pathway was found in certain tropical grasses such as maize and sugar cane (Kortschack et al., 1965, Hatch & Slack, 1970). This pathway, now known to exist in some other kinds

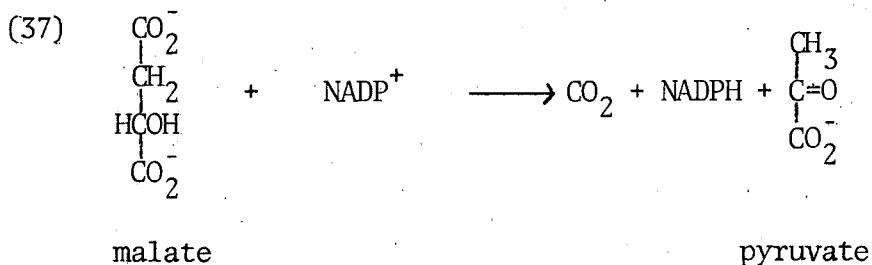
Reductive Pentose Phosphate Cycle



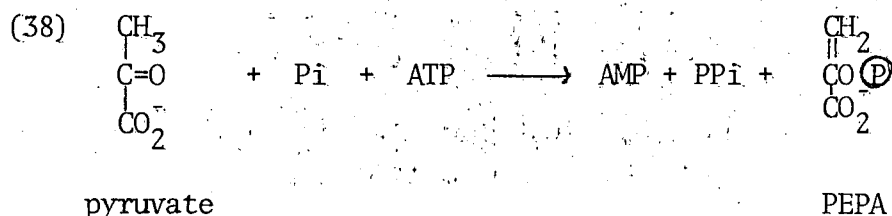
of plants besides tropical grasses, has received much study recently, although some physiological aspects are still unknown. It is generally agreed, however, that the pathway involves the carboxylation of phosphoenolpyruvate (PEPA) to give oxalacetate. This four carbon acid is then reduced with NADPH from the light reactions to give malate, another four carbon acid.



It is thought that this malate is then translocated, either from one kind of green cell to another, or perhaps from cytoplasm into one kind of chloroplast, where it is oxidatively decarboxylated to give pyruvate and CO_2 , as well as NADPH.



This pyruvate is then translocated back to the site of the reaction 36, where it is activated with ATP and inorganic phosphate to give PEPA, pyrophosphate and adenosine monophosphate (AMP). A special enzyme, pyruvate-phosphate dikinase, found only in this type of green plants, mediates this reaction.



The carbon dioxide and NADPH released by reaction 37 are then used for starch and sugar synthesis via the reductive pentose phosphate cycle already described. Thus this cycle represents a preliminary stage of photosynthetic carbon dioxide fixation. It costs 2 molecules of ATP per CO_2 fixed, since the conversion of one ATP molecule to one AMP molecule is equivalent to the conversion of two molecules of ATP to ADP on an energy basis. The plants using this cycle grow in bright sunlight and are not limited by energy, but rather are limited by CO_2 and/or water. The use of this cycle permits the conservation of both water and CO_2 .

While this pyruvate-malate pathway has considerable physiological value to certain plants, it is apparent that it accomplishes little in any artificial pathway from CO_2 to starch, in which energy efficiency is important.

Carboxylation of Acetaldehyde or Acetate

Certain bacteria are able to carboxylate acetaldehyde or reductively carboxylate acetate, in either case giving pyruvate. The difficulties of making acetaldehyde from CO_2 (these apply also to acetate) by organic synthesis have already been mentioned. However, there are biochemical pathways whereby these two carbon compounds might be formed. In either case, however, we come immediately to the problem just mentioned of converting pyruvate to PEPA. There is the extra complexity and energy price of converting pyruvate to PEPA without any other offsetting advantages. There is also the fact that pyruvate-phosphate dikinase is reputed to be a

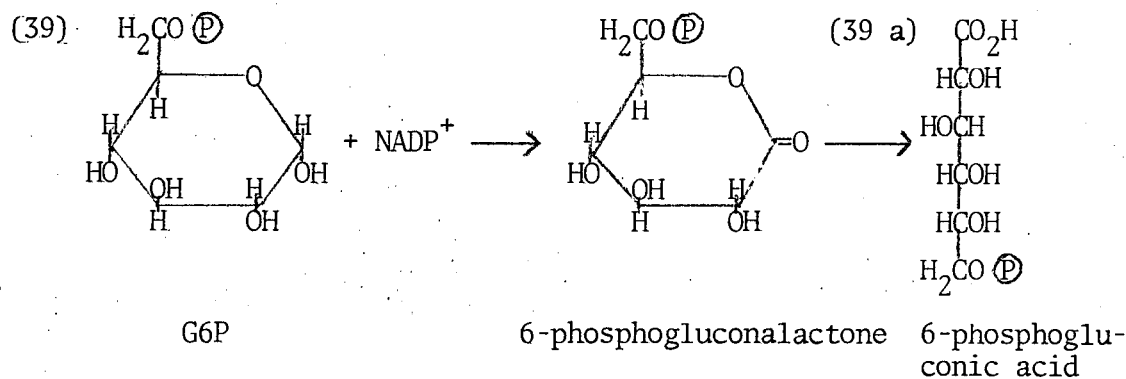
rather unstable enzyme. Pyruvate can also be converted to PEPA via a "shuttle" mechanism involving reductive carboxylation to malate, oxidation to oxalacetate, and decarboxylation to malate in a reaction which uses up a molecule of GTP, the energy equivalent of ATP. This does not seem to be worth the energy and trouble, even if there were efficient routes to acetate or acetaldehyde.

From considerations such as these, it begins to be clear that it is probably better to stick to reactions involving only compounds fairly closely related to sugars, where relatively small changes of functional groups are required. Rather than listing all the various carboxylation reactions we have examined and rejected, we summarize by stating that the system chosen by green plants appears to be the most efficient available, if only enzymically-catalyzed steps are to be used.

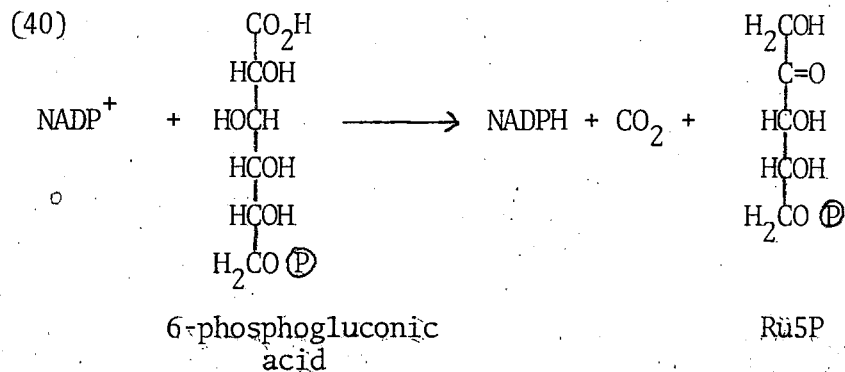
Reversed Oxidative Pentose Phosphate Cycle

Aside from the photosynthetic reductive pentose phosphate cycle for carbon dioxide reduction to sugar, there are two other cyclic pathways involving sugar phosphates which we have considered. One of these is the formaldehyde pathway, already referred to and described below. The other would be a reversal of the oxidative pentose phosphate cycle used by respiring cells (Figure 1).

This oxidative pathway begins with the oxidation of glucose-6-phosphate (G6P) with NADP^+ to give 6-phosphogluconic acid and NADPH.



The second step in this pathway is the oxidation of 6-phosphogluconic acid, again with NADP^+ , to give ribulose-5-phosphate (R5P), CO_2 , and NADPH.

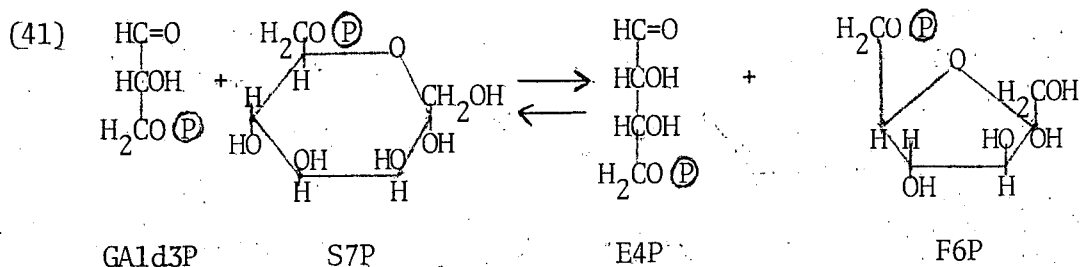


This reaction is reported to be reversible (Horecker and Smyrniotis, 1952). Independent calculations of the free energies of formation of the reactants

and products in this reaction also indicate that the reaction is reversible (Bassham and Krause, 1969).

Once the Ru5P is formed, it is converted back to triose and hexose phosphates via a reversal of many of the reactions involved in the reductive cycle. Thus, two molecules of Ru5P are converted to two molecules of Xu5P. This is a reversal of reaction 32. One molecule of Ru5P is converted to R5P, reversing reaction 33. Then Xu5P and R5P react via a transketolase mediated step to give S7P and GAlD3P, reversing reaction 31.

The next step is mediated by an enzyme, transaldolase, not used in the reductive cycle. In its presence, GAlD3P and S7P are converted to E4P and F6P.



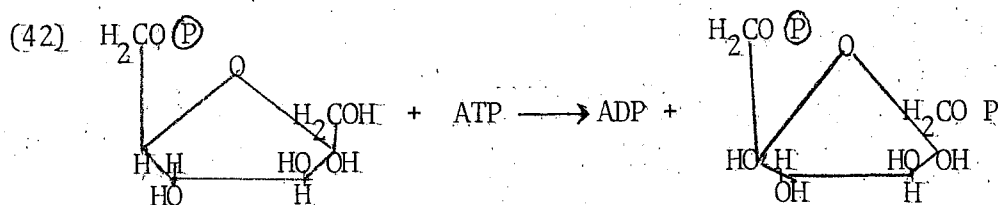
Another transketolase-mediated reaction then converts E4P and the other Xu5P molecule to F6P and GAlD3P. The end result of this sequence of reactions, beginning with Ru5P, is the conversion of three molecules of Ru5P to two molecules of F6P and one of GAlD3P. These could be converted to G6P and the complete cycle repeated. The operation of this cycle thus results in the oxidation of G6P to CO₂ and the reduction of two molecules of NADP⁺ to NADPH for each CO₂ produced.

No ATP is produced from this cycle, and from energy considerations it soon becomes clear that it could be run backwards only if some energy input could be devised. To do this, one must consider which steps in the oxidative cycle are highly irreversible, that is involve large negative

free energy changes in the oxidative direction.

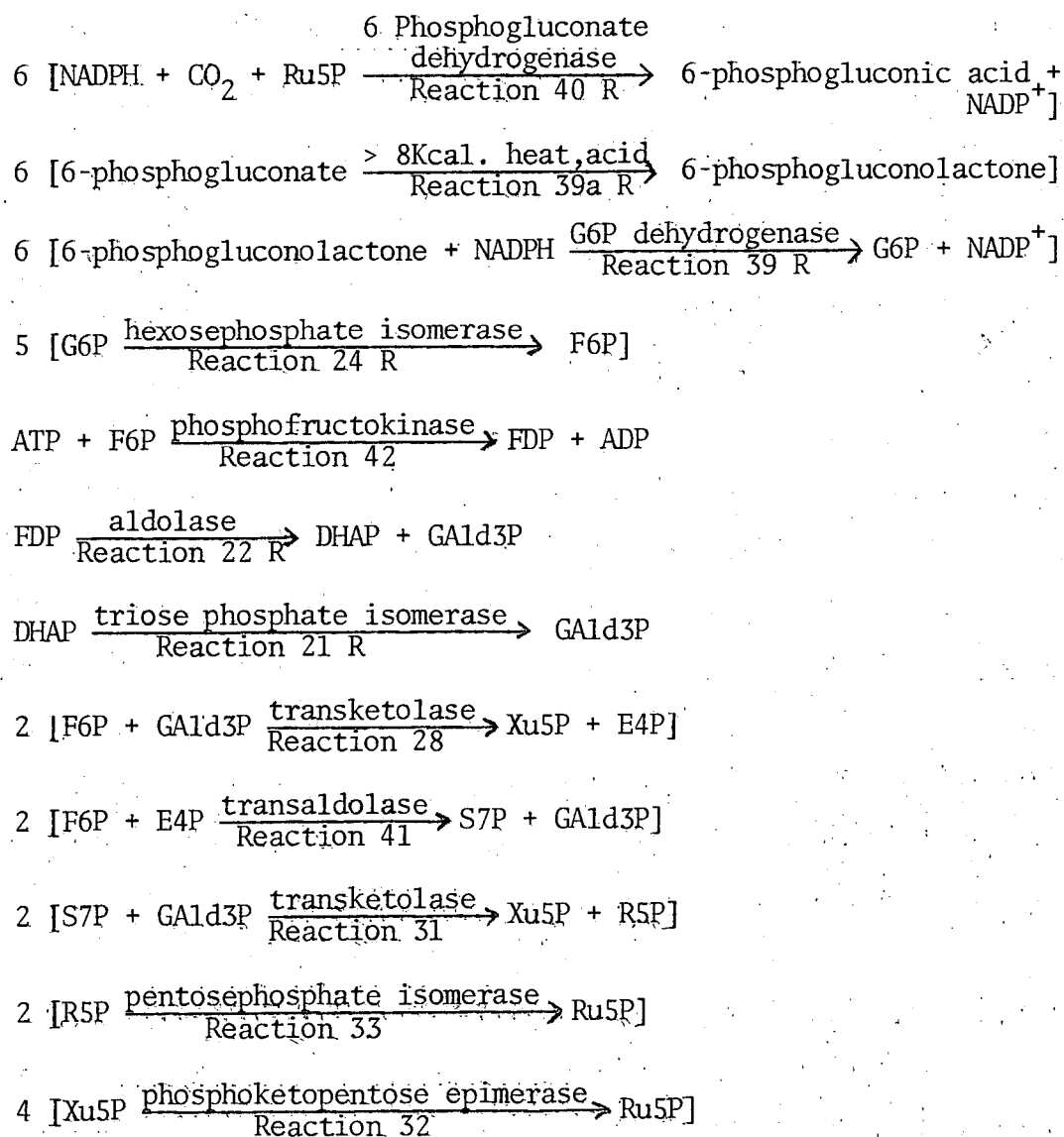
The most important such step is the oxidation of G6P to 6-phosphogluconic acid. As indicated (Reaction 39), this reaction takes place in two steps. The first step is the oxidation of the G6P to 6-phosphogluconolactone, with the conversion of NADP^+ to NADPH. A similar reaction in which glucose is oxidized to gluconolactone with the conversion of NADP^+ to NADPH has been studied (Strecker and Korkes, 1952), and it was found that the reaction to the lactone is highly reversible. The large negative free energy change occurs in the hydrolysis of the lactone to the free acid. It may be assumed that the corresponding free energy changes accompanying the oxidation and hydrolysis of the phosphorylated compounds might be similar. Thus what is needed is a way to provide energy for the conversion of the 6-phosphogluconic acid to its lactone. It is known that some hydroxy-acids can be converted to their lactones with acid and heat. It is not known in what yield this particular lactonization could be effected. If research is to be conducted on promising pathways for the conversion of CO_2 to starch in the future, this lactonization reaction and the subsequent enzymic reduction of the lactone with NADPH would seem to be worthy of study.

Assuming for the moment that this reaction could be carried out efficiently, there is one other point at which an energy input would be required. In order to convert some of the G6P to GAlD3P in the reversed pathway, F6P would have to be converted to FDP with ATP in a reaction mediated by phosphofructokinase.

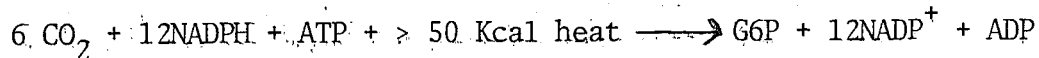


With these reactions for energy input, the reversed path may be visualized according to the following scheme. The end result of this path would be the conversion of carbon dioxide to G6P, using heat and acid, as well as ATP and NADPH. The requirement for ATP would be less than that of the reductive pentose phosphate cycle of photosynthesis. This appears to be the main possible advantage of this path, which will be termed the "Reversed Oxidative Pentose Phosphate Cycle" (See Figure 1, thin lines).

Reversed Oxidative Pentose Phosphate Pathway:



Net Reaction:



If a way can be found to convert 6-phosphogluconate to its lactone efficiently with heat and acid, the overall energy requirement could be less than that of the reductive pentose phosphate cycle. The difficulties and inefficiencies, described later, in regenerating ATP, could give an advantage to the reversed oxidative pentose phosphate cycle which requires only one ATP molecule per G6P formed as compared with 18 ATP molecules required by the reductive pentose phosphate cycle. However, a decision to build a system based on the reversed oxidative cycle could only be made after further research had demonstrated the practicality of reversing the two oxidative steps of the cycle and especially of converting the 6-phosphogluconate to its lactone.

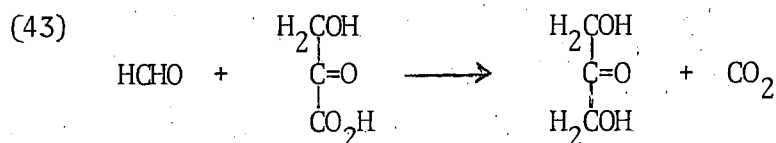
Formaldehyde-Pentose Phosphate Cycle

As mentioned earlier, it is possible to devise a cycle based on the incorporation of formaldehyde. This cycle has the initial disadvantage, already discussed, of requiring 50% more H_2 due to the fact that CO_2 must first be reduced to methanol and then reoxidized. Nevertheless, there are compensating characteristics in this cycle which warrant its serious consideration. As outlined below, the cycle would require several fewer steps than either the reductive pentose phosphate cycle or the reversed oxidative pentose phosphate cycle.

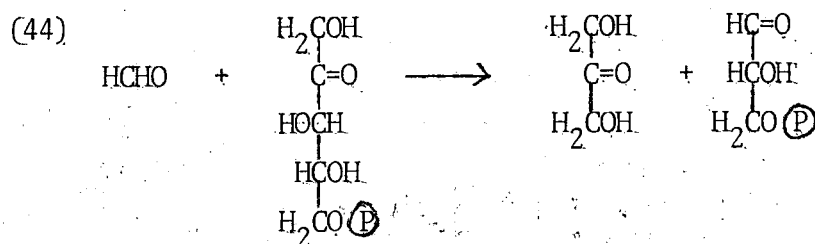
A large uncertainty in the formaldehyde cycle is the initial step. There are apparently rather few biochemical reactions which incorporate formaldehyde directly into sugar phosphates. Kemp and Quayle (1967) described the uptake of formaldehyde by certain bacteria which metabolize

C-1 compounds such as methanol or methane in which the C-1 compounds are converted to formaldehyde which then is incorporated into sugar phosphates. Kemp and Quayle (1966) reported that a pentose phosphate cycle operates to regenerate a pentose phosphate formaldehyde acceptor. In their scheme, formaldehyde was condensed with ribose-5-phosphate to give allulose-6-phosphate, a 6-carbon sugar monophosphate. They proposed that this sugar phosphate was converted to fructose-6-phosphate, after which rearrangements via enzymes of the pentose phosphate cycle regenerated the ribose-5-phosphate acceptor. However, further study of this system has revealed that the reactions are not quite as thought (Quayle, 1972). In any event, the enzyme involved in the formaldehyde incorporation is difficult to isolate, and its reaction not yet well-characterized.

Of perhaps more interest is a report (Dickens and Williamson, 1958) of the participation of formaldehyde in reactions mediated by transketolase. The reaction studied was between formaldehyde and hydroxypyruvate, and the products were CO_2 and dihydroxyacetone in an irreversible reaction.

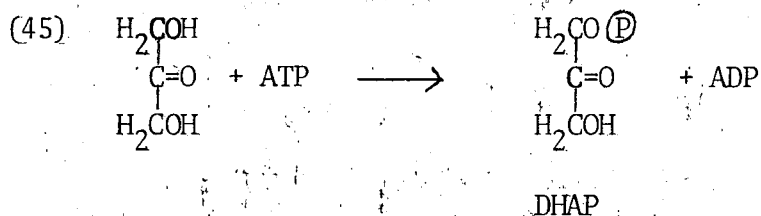


If transketolase could mediate a reaction between formaldehyde and Xu5P, the products would be GAld3P and dihydroxyacetone.



We could find no report of such a reaction in the literature. Letters to Professors Dickens and Racker answers suggesting that the transketolase reaction with formaldehyde may not have been further studied. Thus the following scheme based on this reaction must remain tentative. Nevertheless it is given because it represents a considerable simplification of the enzyme-mediated path to carbohydrate.

A reaction mediated by triokinase would convert dihydroxyacetone to its phosphate, DHAP, with ATP.

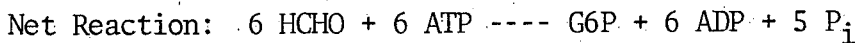
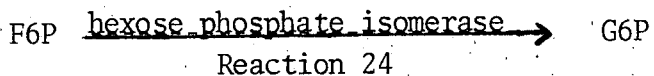
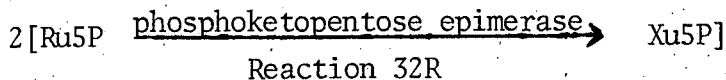
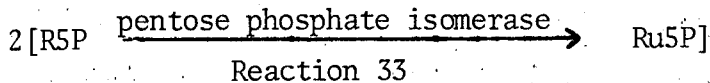
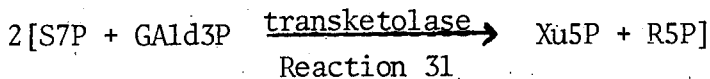
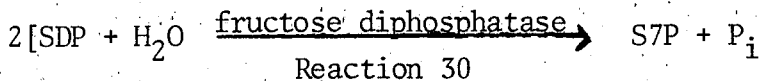
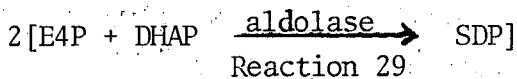
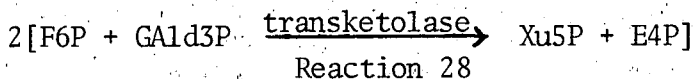
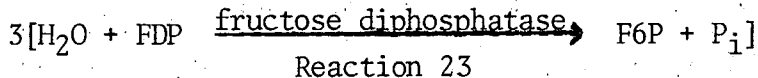
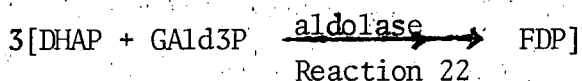
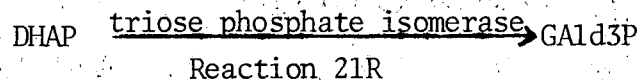
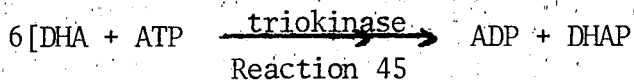
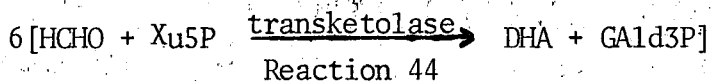


For each three pairs of triose phosphates formed (six triose phosphates), one DHAP would be converted with triose phosphate isomerase (Reaction 21 R) to GAlD3P, so that the net result of the three transketolase incorporations of formaldehyde would be two DHAP molecules and four GAlD3P molecules.

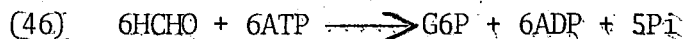
The conversion of five molecules of triose phosphate to three molecules of pentose phosphate would follow just the same pathway as in the reductive pentose phosphate cycle, except that the final product would be Xu5P instead of Ru5P. Two of the pentose phosphate molecules made by the transketolase reactions are Xu5P to begin with, and the R5P could be converted to Xu5P via Ru5P (Reactions 33 and 32 R).

The reactions of these three Xu5P molecules with formaldehyde, and subsequent steps produce six molecules of triose phosphate, one more than needed to regenerate the Xu5P molecules. Each two complete cycles would thus form two triose phosphate molecules from which a net of one G6P would be made.

Formaldehyde - Pentose Phosphate Cycle



The overall net reaction, starting with formaldehyde would be:



This requirement for 6 ATP molecules (1 ATP per HCHO) compares with 18 ATP molecules for the reductive pentose phosphate cycle and 1 ATP for the reversed oxidative pentose phosphate cycle. The formaldehyde cycle requires no reductive reactions, and this would eliminate the need for a system to reduce NADP^+ to NADPH. To calculate overall energetics, one must include the three moles of H_2 needed to reduce CO_2 to formaldehyde via methanol. Starting with H_2O , 3 moles of H_2 costs $3 \times 56.7 = 170.1$ Kcal. Adding 1 ATP (12 Kcal) gives a total chemical energy requirement of 182 Kcal. However, if the regeneration of ATP is very inefficient, the total energy cost could be less than in the case of the reductive pentose phosphate cycle.

Comparison of Three Pathways

We are now in a position to compare the three principal pathways we have discussed. There may be other pathways, but we reiterate our contention that pathways involving direct incorporation of one-carbon compounds into carbohydrate-like compounds seem likely to be the most efficient. It will be recalled from our earlier discussion, that organic syntheses of two-carbon compounds appeared to require too much energy. Biochemical incorporation of carbon into non-carbohydrate compounds must be followed by a large number of biochemical steps to convert such compounds to carbohydrate.

Of the three pathways discussed above, only the photosynthetic carbon reduction pathway, the reductive pentose phosphate cycle, is known to occur as written in nature. All of its enzymes are together in the same physiological environment in the chloroplast. Thus, their ability to function in a common environment of pH, metabolite concentrations, Mg^{++} ion concentration,

etc. is assured. This could be a great advantage if it develops that the best system for reactors leading to starch synthesis is a multienzyme reactor. Considering the magnitude of the separations of products and reactants involved, there seems to be a high probability that such a multi-enzyme system may be advantageous.

Of course, all of the enzymes of the reversed oxidative pentose phosphate cycle also occur together. However, it would be necessary in the case of that cycle to isolate the 6-phosphogluconic acid prior to its conversion to 6-phosphogluconolactone. The fact that 6-phosphogluconic acid is the only carboxylic acid in the cycle is not all that helpful, since most of the other metabolites are phosphates, and hence also anions at neutral pH.

The formaldehyde pentose phosphate cycle would be significantly simpler than the other cycles, since it substitutes the transketolase reaction, the enzyme which is required in all three cycles, for the carboxylation reaction, and the reductive reactions of the other cycles. It has its own kinase reaction, but so do the other cycles, the reductive pentose cycle requiring two kinase reactions with two different enzymes.

Weighing against this advantage are three serious disadvantages: (1) A requirement of 50% more H_2 , and (2) Our present lack of certainty that the transketolase reaction with formaldehyde and Xu5P would work. (3) If formaldehyde would react with Xu5P, it might also react with F6P to give dihydroxyacetone and E4P, and with S7P to give dihydroxyacetone and R5P. Since these are also intermediates in the proposed cycle, a scheme could perhaps be worked out to use such reactions, but some problems of control might ensue. More serious would be a reaction of formaldehyde with dihydroxyacetone phosphate to give erythrulose-4-phosphate. This reaction has been

reported (Charalampous and Mueller, 1953). Another enzyme would then have to be found to convert erythrulose-4-phosphate to E4P, an intermediate compound of the cycle.

There is also the danger that formaldehyde, a fairly reactive chemical, might poison some of the enzymes, if a multienzyme system were employed.

Given these uncertainties about the interaction of formaldehyde with the enzymes discussed, plus the higher initial energy input to convert CO_2 to formaldehyde via methanol, we are unable to select the formaldehyde path as our first choice. However, it would appear that this pathway could become the one of choice if a way could be found to reduce CO_2 directly to formaldehyde, and the enzymology should prove to be favorable after further study.

The reversed oxidative pentose phosphate cycle offers a possible advantage over the reductive pentose phosphate cycle in terms of a large saving on ATP input. Depending on how efficiently the 6-phosphogluconic acid could be converted to 6-phosphogluconolactone and how much energy must be expended to regenerate ATP from ADP and inorganic phosphate, this could be a very important factor. Since both of these factors are unknown, it is impossible to make a decision on this basis.

A severe disadvantage of the reversed oxidative pentose phosphate cycle is our lack of knowledge about the feasibility of reversing the two oxidative steps. From a thermodynamic standpoint, both steps are feasible, if the acid can be chemically converted to the lactone. Overall, this cycle has about the same number of steps as the reductive pentose phosphate cycle. Since this cycle has not been run backward, and since we are not certain that the acid can be efficiently converted to the lactone, we feel we must make the reductive pentose phosphate cycle our first choice.

The reductive pentose phosphate cycle is the unique biochemical pathway, selected by evolution, for the conversion of CO_2 to starch. It seems probable that no better pathway can be devised to operate under the conditions existing in a living cell. This does not rule out the possibility that a more efficient pathway can be devised using conditions not tolerable in a living cell. Also, the living cell enjoys advantages that make for great efficiency. In particular, all the reactions of the reductive pentose phosphate cell take place within an organelle only 5 micrometers in diameter. Thus, the complexity of the cycle doesn't cause any diffusional problems. Another great advantage of the living cell is the great selectivity of permeability of transport of chemicals through the limiting membrane of the subcellular organelle, the chloroplast.

From such considerations, it clearly is not justified to conclude that what is best for the microbiochemical factory of the green plant cell is necessarily best for the macrochemical factory built by man. But perhaps because of the lack of known facts about key features of some possible alternative pathways, we have been unable to find such a pathway which is demonstrably more efficient than the photosynthetic pathway.

From a purely chemical standpoint the reductive pentose phosphate cycle is remarkably efficient. Since the starting point for our proposed starch synthesis is water and CO_2 , it is fair to calculate efficiency on this basis. The energy stored in converting one mole of CO_2 and one of water to a mole of O_2 and a sixth mole of glucose is +114.4 Kcal (Reaction 1). The energy requirement of the cycle per CO_2 mole incorporated is two moles of H_2 and three moles of ATP (Reaction 35). Splitting two moles of water to give two moles of H_2 and one of O_2 cost 113.4 Kcal (Equation 2), while three moles of ATP is worth about 36 Kcal. Thus the overall efficiency of the reductive

pentose phosphate cycle may be calculated as $114.4/149.4 = 75\%$. Of course, the cost of producing the ATP outside of the cell may be much higher than it is in the cell (where it costs about 56 Kcal to store 36 Kcal as ATP).

One other advantage of the reductive pentose phosphate cycle may be mentioned. If a factory based on stabilized enzymes is to be built, it is important to have a source of abundant, inexpensive materials to supply the enzymes, since they appear likely to have a limited life, even in the stabilized state. All of the enzymes of the reductive pentose phosphate cycle are of course present in all green leaves.

Recommended Pathway

A principal purpose of this study was to examine possible pathways for the conversion of CO_2 to starch, using energy and water, and to recommend the optimal pathway. At the outset, we had expected to select the most promising several pathways on the basis of simplicity, energy efficiency, and probability that the pathway could provide a successful route in terms of practicality of the reactions and of the engineering concepts necessary to convert the reactions into useful industrial steps. We had expected that it would be possible to incorporate steps of organic synthesis, not involving the use of enzymes with their attendant difficulties, and to reserve the enzymes for those later steps in the pathway in which stereospecificity of the products must be maintained.

At the same time, we had expected that there might be diverse biochemical steps from different systems that could be incorporated into the pathway to give a system more efficient than any found in nature. We didn't really expect that this would be the case if all the steps were to be enzymically catalyzed, for evolution has had a long time to perfect the best such system. However, if some organic reactions carried out under

non-physiological conditions were included, it could change the rules of the game, and other pathways could become more efficient.

As it turns out, we are forced to make a choice in favor of the photosynthetic carbon reduction path--the reductive pentose phosphate cycle. This choice is dictated partly by ignorance in the literature about key steps in alternative pathways and partly by ignorance about organic reactions for converting carbon dioxide to reduced carbon compounds with a high energy efficiency. Organic chemists have not been faced with this problem. In our present petrochemically-based technology of organic compounds it is much cheaper to begin reaction sequences with already reduced organic compounds. Those chemists who have been faced with the problem of organic synthesis with CO_2 , say, in the synthesis of ^{14}C -labeled organic compounds, have not been under any constraints as to the use of energy or energy-rich compounds such as lithium aluminum hydride.

Problems and Possible Solutions

Having chosen the reductive pentose phosphate cycle, we must consider its disadvantages and how they may be overcome. The first obvious disadvantage is the carboxylation reaction itself. As the later sections will show, the carboxylation enzyme, ribulose diphosphate carboxylase, is a high molecular weight protein, rather low in turnover number, somewhat unstable, and probably subject to complex regulatory mechanisms in vivo. This enzyme is known to require CO_2 , not bicarbonate ion, as its substrate. Thus in any liquid system in which a large portion of the carbon is in the form of bicarbonate ion it might be necessary to include some carbonic anhydrase, an enzyme that speeds the equilibration between CO_2 and carbonic acid. However, this requirement can probably be obviated by using higher

concentrations of CO_2 , and it may be an engineering advantage to be able to do just that. As discussed under Properties of the Enzymes, lower molecular weight forms of the carboxylase are found in certain bacteria, and this could be an advantage in plant design. Beyond this we can only suggest extensive further research on the properties of the carboxylation enzyme, and on its insolubilized and stabilized form.

Another difficulty with the reductive pentose phosphate cycle is its large number of steps. The cycle itself contains 13 steps, although 10 enzymes are required, since 3 enzymes, aldolase, transketolase, and FDPase-SDPase do two jobs each. Another 5 enzymes are required to reach starch in nature. If starch phosphorylase can be substituted for the four steps between GlP and starch, this number would be reduced to two.

As will be discussed in the engineering sections of this report, there are formidable problems of separation of metabolites, coenzymes, inorganic ions, etc., associated with the operation of a reactor representing any of these steps catalyzed by enzymes insolubilized on particles in a fixed bed. If such separations are to be carried out for each of the 15 to 20 possible enzymically-catalyzed steps (if we include regeneration of ATP and NADPH), the plant costs and energy requirements will multiply enormously. The energy cost could easily become one to two orders of magnitude greater than the chemical energy storage accomplished. Even though we are assuming a supply of inexpensive energy (without which this system could never become practical) waste of energy on a vast scale probably will not be tolerable.

In order to minimize this waste of energy due to separation processes, it appears possible that most, if not all, of the enzymically-catalyzed

steps should be carried out in a common "stirred-tank" reactor. Such a system may approach on a macro scale what the chloroplasts accomplish on a micro scale. Thus, perhaps the only inputs to the reactor could be CO_2 , H_2 gas, and acetyl phosphate. The outputs could be acetate and phosphate and starch. A portion of the reactor fluid would be continuously filtered and cycled through starch-synthesizing columns. Starch would collect on these columns, which would be removed from time to time for separation of the starch from the column material. We know that all of the components of the reductive pentose cycle (enzymes, metabolites, etc.) are compatible. We are not certain that the enzymes which would be involved in the transfer of electrons from H_2 to ferredoxin to NADP^+ are likewise compatible with all the rest. There is some reason to hope that they would be, since certain algae can be adapted to carry out a dark reductive pentose phosphate cycle using H_2 instead of light.

Certain other advantages may be cited for the system just described. First, the presence of H_2 gas in the system (and the exclusion of O_2) would probably increase the stability of most enzymes.

Second, the H_2 atmosphere, anaerobic, would greatly reduce the probability of serious bacteriological contamination. Very few species of bacteria live on H_2 in the absence of O_2 . Fermentative bacteria would still be a threat, but their growth might be inhibited by the presence of acetate and the absence of very much free sugar. Also, antibiotics could be used.

Third, the system would be to some extent self regulating. Three of the enzymes catalyze more than one step, and these enzymes would tend to catalyze the reaction for which there was the higher level of substrate, taking into account the binding constants for the substrates. By making

certain enzymes rate-limiting (as they are in the chloroplasts) and monitoring and controlling the amounts of these key enzymes, a high degree of regulation could be achieved with relatively few controls.

Fourth, since all steps would take place in the same reactor, and since only end products would be removed, all reactions would eventually go to completion. There would be no need for any recycling except in the final stage of starch synthesis.

The problems of running all steps in the same reactor are not insurmountable. Diffusion times could be reduced perhaps by having the enzymes for several sequential steps attached to one bead. For example, beads could be prepared with pentose phosphate isomerase (Reaction 33), phosphoketopimerase (Reaction 32), phosphoribulokinase (Reaction 34), and ribulose diphosphate carboxylase (Reaction 15).

In summary, I am recommending that the reductive pentose phosphate cycle be used, with an input of H_2 , CO_2 , and acetyl phosphate, and that all steps be carried out in a single multicomponent reactor. Such a reactor would be a kind of macrochloroplast, although without the light-harvesting apparatus.

Energetics of the Reductive Pentose Phosphate Cycle and Alternate Cycles

The "physiological" standard free energy changes ($\Delta G^{\circ'}$) of the reactions of the reductive pentose phosphate cycle have been calculated by Bassham and Krause (1969). The standard conditions for these physiological free energy changes are that all activities of solutes are at unity and all gases at 1 atmosphere, but $[H^+] = 10^{-7}$.

The actual concentrations of metabolic intermediate compounds in photosynthesizing Chlorella pyrenoidosa, and from these and the $\Delta G^{\circ'}$ values, the steady-state (ΔG^S) values, in that system, were calculated. The ΔG^S values

would, of course, vary with the system. However, they are of some interest in the present context. There is a limited range of concentrations that is optimal for the enzymes, particularly in a multienzyme system, where one enzyme may be exposed to the reactants and products of another enzyme-mediated reaction. Thus it is likely that, if a multienzyme system is employed in a reactor of the starch synthesis plant, concentrations within an order of magnitude of those found in the natural system of green plant cells will be required. The ΔG^S values would consequently not be too different in the artificial system.

For example, inorganic phosphate, P_i , is commonly in the range of concentration of 1 mM in plant cells. This adds -4.2 Kcal to the ΔG values of reactions in which P_i is liberated (Reactions 23 and 30).

Table I shows the $\Delta G^{O'}$ and ΔG^S values for reactions of the reductive and oxidative pentose phosphate cycles. Table II shows free energies of formation from the elements of metabolites. From an examination of Table I several points are apparent:

- 1) ΔG^S values are generally close to zero for the majority of reactions, indicating high reversibility.

- 2) A few reactions have larger negative values for ΔG^S . In the reductive cycle these are particularly the carboxylation reaction and the FDPase-mediated reactions.

- 3) In order for the reduction of PGA to triose phosphate to occur (have a ΔG^S with a negative value), the concentrations of reactants (ATP, NADPH, and PGA) must be considerably higher than the concentrations of the products (GAPd3P, $NADP^+$, P_i , and ADP). However, the fact that there are 3 reactants and 4 products helps, since the concentrations of all metabolites lie within an order of magnitude of 2×10^{-4} M.

Table I. Free Energy Changes for Reactions of the Reductive and Oxidative Pentose Phosphate Cycles

| Reaction No | Reactants | Products | G ^{0'} Kcal | G ^S Kcal |
|-------------|--|--|-------------------------|------------------------|
| 15 | RuDP, CO ₂ , H ₂ O | 3-PGA | -8.4 | -9.8 |
| 16 | ATP, 3-PGA | ADP, PPGA | | |
| 17 | PPGA, NADPH | GA1d3P, NADP ⁺ , P _i | +4.3 | -1.6 |
| 21 | GA1d3P | DHAP | -1.8 | -0.2 |
| 22 | GA1d3P, DHAP | FDP | -5.4 | -0.4 |
| 23 | FDP, H ₂ O | F6P, P _i | -3.4 | -6.5 |
| 24 | F6P | G6P | -0.5 | -0.3 |
| 25 | G6P | G1P | +1.7 | - |
| 28 | F6P, GA1d3P | E4P, Xu5P | +1.5 | -0.9 |
| 29 | E4P, DHAP | SDP | -5.6 | -0.2 |
| 30 | SDP, H ₂ O | S7P, P _i | -3.4 | -7.1 |
| 31 | S7P, GA1d3P | R5P, Xu5P | +0.1 | -1.4 |
| 32 | Xu5P | Ru5P | +0.2 | -0.1 |
| 33 | R5P | Ru5P | +0.5 | -0.1 |
| 34 | Ru5P, ATP | RuDP, ADP | -5.2 | -3.8 |
| 39,39a | G6P, NADP ⁺ | 6-phosphogluconate, NADPH | -8.4 | -11.4 |
| 40 | 6-phosphogluconate, NADP | Ru5P, NADPH, CO ₂ | +1.6 | -2.1 |

From Bassham & Krause, 1969.

Table II. Physiological Free Energies of Formation from the Elements
of Metabolites and Related Compounds

| Substance | G° Kcal (1 M, aq) |
|------------------------------|----------------------------------|
| $P_i^{-2}(-H_2O)$ | P |
| α -D-glucose | -219.2 |
| G6P $^{-2}$ | P-215.9 |
| G1P $^{-2}$ | P-214.2 |
| F6P $^{-2}$ | P-215.4 |
| FDP $^{-2}$ | 2P-212.0 |
| DHAP $^{-2}$ | P-104.3 |
| GAld3P $^{-2}$ | P-102.5 |
| Glycerol | -116.8 |
| α -glycerol P $^{-2}$ | P-114.4 |
| 3-PGA $^{-3}$ | P-157.5 |
| 2-PGA $^{-3}$ | P-156.1 |
| pyruvate $^{-1}$ | -113.4 |
| E4P $^{-2}$ | P-139.1 |
| S7P $^{-2}$ | P-252.5 |
| SDP $^{-4}$ | 2P-249.1 |
| R5P $^{-2}$ | P-177.6 |
| Ru5P $^{-2}$ | P-177.0 |
| RuDP $^{-4}$ | 2P-174.6 |
| Xu5P $^{-2}$ | P-177.3 |
| 6-phosphogluconate | P-267.4 |
| CO ₂ | -94.3 |
| H ₂ O | -56.7 |
| H ⁺ | -9.6 |

The high degree of reversibility of many steps may well be a strong argument in favor of a multienzyme reactor. For example, if all enzymes of the reductive pentose phosphate cycle were present in a single reactor, the large negative ΔG^S of the carboxylation reaction (-10 Kcal) would drive carbon into the reaction pathway. Subsequent reversibility would not matter, since carbon would be removed from the system in the starch-forming reaction, which also is estimated to have a substantial negative free energy change (assuming the ADPG system, Reactions 26 and 27, is used!).

If a single enzyme, fixed-bed column were used for a highly reversible step, it is clear that considerable unreacted material would come out of the bottom of the column, and would have to be separated from the reactants, coenzymes, etc. Separation problems are severe in terms of plant size, energy consumption, and cost, and must be minimized wherever possible.

This is another reason for seriously considering a single, multienzyme system suggested earlier, in which the only inputs are CO_2 , H_2 , and acetyl phosphate. The only outputs would be acetate, phosphate, and starch. The starch might be removed on a side-loop column, where it could be collected in insoluble form. The column (one of many) could be removed, and the starch washed off (perhaps following partial enzymic digestion). The acetate and phosphate, having lower molecular weights than other metabolites and coenzymes, would be removed in other loops employing a battery of counter-current selective diffusion devices, as described in the engineering reports.

While the example in the engineering report describes the separation of different metabolites, it is hoped that the principle could be used for separating acetate and phosphate from other metabolites. It is clear, however, that considerable improvement in the state of the art of selective diffusion membranes will be required before this hope becomes a reality.

Assuming that the low cost energy postulated as the basis of this study is achieved, the single most serious obstacle to success appears to be the separation problems. We might summarize this opinion by saying that the use of insolubilized enzymes appears to offer the promise of duplicating in vivo catalysis, but membrane technology is much further at the present time from duplicating in vivo selective transport. Both of these properties of living cells will have to be copied in some way if we are to reproduce the synthetic process of photosynthesis.

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PART II

Enzymes and Their Properties

Lydia Bearden

INTRODUCTION

One of the criteria in selecting the optimal pathway for the synthesis of starch from CO_2 , water and energy had to be the number and characteristics of the enzymes involved. In turn, this assumes the availability of information on these enzymes. The present section summarizes the literature available on the enzymes participating in the reductive pentose phosphate cycle--the pathway which was selected as our first choice. Perhaps after scanning through this information the reader might wonder why we selected this particular pathway. Indeed, there are very large gaps in our knowledge of the enzymes. One can only reply that the problems and lack of information appeared even greater in the other alternatives considered. Many examples of such problem areas were given in previous sections of the report.

The enzymes in the following survey are arranged alphabetically and are also numbered according to the number given in previous sections to the reaction they catalyze. Although we have tried to be fairly complete in the bibliography, the summaries themselves concentrate mainly on the data which we felt would be needed for a systems design. These are principally data on the enzyme at the molecular level such as molecular weights, presence of cofactors, pH optima, kinetic parameters, data on inhibitors, etc. Any available information on stability and primary structure was at least referred to, as a basis for possible future research on the insolubilization and stabilization of enzymes.

What are some of the overall conclusions emerging from this survey of enzymes? It is clear that much work is still needed on the enzymes themselves. In a few cases (phosphoglycerate kinase, ribulose phosphate

epimerase, phosphoribulokinase) it will be seen that few or no highly purified preparations of the enzyme have as yet been described. In other cases (glyceraldehyde 3-phosphate dehydrogenase, transketolase, triose phosphate isomerase, aldolase, fructose diphosphatase) the enzymes are well known, but they have been studied mostly from the point of view of the mammalian biochemist, i.e., as degradative enzymes participating in such metabolic pathways as glycolysis and oxidative pentose phosphate. These enzymes were, generally speaking, purified from yeast or mammalian tissues rather than from plants, and in many cases there are few or no available data for the reactions performed in the direction of the synthetic reductive pentose phosphate cycle.

We would have liked to have more data on the plant enzymes, as these might be more useful in the starch synthesis for two main reasons:

- 1) First, plants would be a cheaper source of enzyme and would contain all the enzymes needed for the pathway of interest. (Two of the enzymes in the cycle, ribulose diphosphate carboxylase and phosphoribulokinase, are unique to the reductive pentose phosphate cycle.)

- 2) Secondly, we have no information on the in vitro compatibility of the various enzymes needed, but we know at least that all the enzymes are able to function together in their natural environment--the chloroplast. It likely would be easier to find conditions of pH, ionic strength, etc. suitable for all the enzymes involved, or at least for a majority of them, if they were purified from a common source. This would be important for a design such as is proposed in the section on pathway selection, in which a number of reactions are performed in a common reactor. It must be stressed, however, that it is impossible to predict on the basis of available data

whether this is a realistic approach to the problem from a chemical point of view. Not only is the information lacking on the in vitro compatibility of various soluble enzymes, but we also have to contend with the fact that the properties of the enzymes might change considerably during the process of insolubilization. Virtually no work is available on the insolubilization of the particular enzymes needed. Therefore, at the present time, any design would have to be based on whatever information is available on the soluble enzymes, and this will almost certainly introduce a large degree of uncertainty.

This section also includes a summary of the available information on enzyme insolubilization and stabilization. The work that has been done on other enzymes might at least serve to make some reasonable predictions about such required parameters as the amount of protein bound per unit of support and the proportion of protein remaining enzymatically active after binding.

In conclusion, it can be stated that much research is still needed in the following areas:

- 1) Purification of all the enzymes involved using green plants as a common source.

- 2) Characterization of the enzymes at the molecular level, especially from the point of view of the reactions as they occur in the reductive pentose phosphate cycle. This involves in a number of cases the developing of new assay procedures measuring enzyme activity in the direction needed.

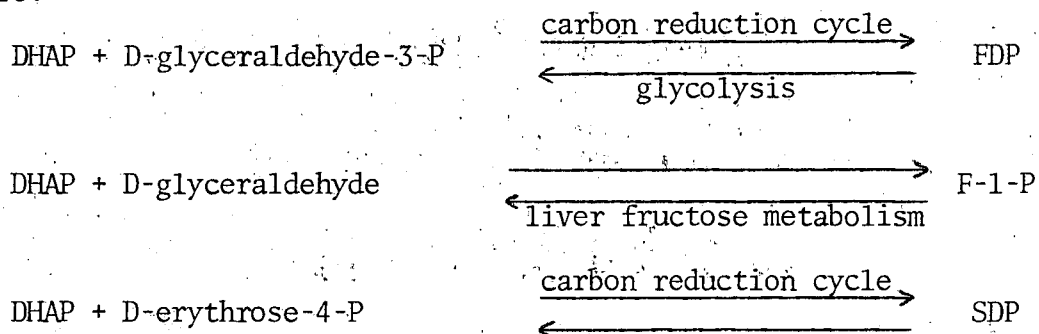
- 3) Insolubilization and stabilization of the enzymes, elucidation of the properties of the derivatives and their compatibility. This area is certainly the biggest question mark at the present time.

Survey of Enzymes

ALDOLASE

(Reactions 22, 29)

"Aldolase" is a generic name for a number of enzymes catalyzing the aldol cleavage/condensation reactions involving dihydroxyacetone phosphate and a variety of aldehydes. Among these reactions, the most important ones are:



A trans configuration at positions 3 and 4 is required.

Aldolases are present and have been studied in representatives from all phyla. A partial bibliography on the enzyme comprising mainly the more recent work is compiled at the end of this section. It includes several reviews dealing with various particular aspects of the enzyme: Rutter (1961), Rutter (1964), Morse and Horecker (1968), Horecker (1971). In spite of the wealth of available studies, the data most necessary for our purpose are very scarce or apparently not available. This is because aldolase has been studied mostly as the glycolytic enzyme, the assays being performed in the direction of FDP cleavage. Thus data on FDP synthesis are seldom given and data on SDP formation do not appear to be available.

Aldolases are classified in two large groups (Rutter, 1964): class I and class II. These are sufficiently different from each other to justify the assumption that they are analogous (rather than homologous) proteins.

I. Class I aldolases

The prototype for this class is the mammalian muscle aldolase. The group includes a number of mammalian aldolase isozymes from different organs, enzymes from various other animal phyla, as well as the enzyme from plants.

The distinguishing features of this class are: a molecular weight of 140,000 - 160,000, no inhibition by metal chelators, no bound metal ion, no activation by monovalent ions, and broad pH optima. Data on three representatives of this class are compiled in Table I.

It should be noted from the kinetic parameters of the two mammalian isozymes that aldolase B (liver) appears to be adapted for fructose metabolism and gluconeogenesis, i.e. for FDP synthesis. Therefore, of the two mammalian aldolases described, aldolase B would be more suitable for the sugar synthesis process. Unfortunately, the kinetic parameters for the spinach enzyme were studied in the direction of cleavage both of FDP and of SDP, even though these are the inverse of the postulated photosynthetic reactions. Moreover, data for the reaction of SDP synthesis from E-4-P and DHAP seem to be unavailable for any of the enzymes studied.

II. Class II aldolases

This group is found in fungi and bacteria (including the blue-green "algae") and is distinguished by a molecular weight of about 70,000, the presence of a bound divalent metal ion, complete inhibition by EDTA (reversible by addition of divalent metal ion), activation by K^+ , and sharper pH optima. In contrast to class I enzymes, class II aldolases are unaffected by carboxypeptidase and are inhibited by reagents which attack -SH groups. These differences suggest the existence of two different mechanisms of action and there have been a large number of studies addressing

Table I. Properties of Some Class I Aldolases *

| | Rabbit Muscle (Aldolase A) | Rabbit Liver (Aldolase B) | Spinach |
|---------------------------------------|-------------------------------|------------------------------|----------------------|
| Molecular weight | 160,000 | 150,000 | 142,000 |
| Subunits | 4 | 3 ? | ? |
| Inhibition by EDTA | 0 | 0 | 0 |
| V_{\max} FDP synthesis [‡] | 10,000 | 3,000 | ? |
| V_{\max} FDP cleavage | 5,300 | 460 | 3,900 |
| V_{\max} FIP cleavage | 105 | 460 | 156 |
| V_{\max} SDP cleavage | 5,400 | - | 2,300 |
| K_m (M) | | | |
| FDP | 6×10^{-5} | 2×10^{-6} | 6.8×10^{-5} |
| FIP | 4×10^{-3} | 8×10^{-4} | 3.9×10^{-3} |
| DHAP | 2×10^{-3} | 4×10^{-4} | ? |
| GAP | 1×10^{-3} | 3×10^{-4} | ? |
| SDP | 3.1×10^{-5} | ? | 1.7×10^{-5} |
| pH optimum | 6.9 - 8.8 | - | 7.5 - 8.25 |

* Compiled from: Rutter (1961 and 1964), Morse and Horecker (1968), Brooks and Criddle (1966).

[‡] V_{\max} units: mole substrate/minute per mole protein.

themselves to the question of the mechanism of the aldolase reaction and the differences between the two groups. (For a review see Morse and Horecker, 1968).

For the yeast enzyme Rutter (1961) gives the following kinetic constants:

$$K_m \text{ (FDP)} = 3 \cdot 9 \times 10^{-4} \text{ M}; \quad V_{\max} = 8300$$

$$K_m \text{ (DHAP and GAP)} = 2 \times 10^{-3} \text{ M}.$$

An enzyme of the class II type that might deserve special mention is that from the thermophilic bacteria (Barnes et al., 1970; Sugimoto and Nosoh, 1971) which is both active and stable at high temperatures.

The following thermodynamic constants for the aldolase reaction were compiled by Rutter (1961):

| | ΔG° (cal/mol) | ΔH° (cal/mol) | ΔS° (cal/deg.mol) |
|--|----------------------------|----------------------------|--------------------------------|
| FDP \longrightarrow DHAP + GAP | 5580 | 13,100 | 24 |
| $K_{\text{eq}} = 8.1 \times 10^{-5} \text{ M (30}^\circ\text{)}$ | | | |
| F-1-P \longrightarrow DHAP + glyceraldehyde | 7880 | 15,000 | 23 |

Recently, a number of studies have addressed themselves to the elucidation of the primary structure of aldolases from various organisms, especially around the active center. (See for example, Horecker, 1971; Guha et al., 1971; Ting et al., 1971a and 1971b; Lai et al., 1971; Lai and Oshima, 1971; Jack and Harris, 1971).

Rabbit muscle aldolase has been insolubilized by Bernfeld et al. (1968) by imbedding it in polyacrylamide gel. In this work only about one fifth of the protein fixed remained enzymatically active and the authors suggested that only the surface-bound protein retained activity.

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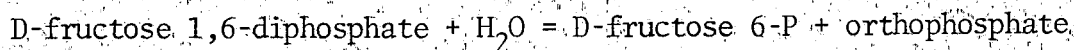
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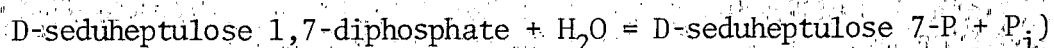
FRUCTOSE-1,6-DIPHOSPHATASE (HEXOSEDIPHOSPHATASE)

(D-Fructose-1,6-diphosphate 1-phosphohydrolase; E.C. 3.1.3.11)

(Reactions 23, 30)



(Note: This section includes comments on "Seduheptulose 1,7-diphosphatase".



FDPase* is a key enzyme in both gluconeogenesis and the photosynthetic carbon reduction cycle. It is thought to be a regulatory enzyme in both these systems and, as such, has been extensively studied in the past few years. To summarize its properties in just a few pages is therefore a very difficult task. Pontremoli and Horecker have recently (1970 and 1971) written two detailed reviews on FDPase, with numerous references and the present summary is based mostly on these reviews and the work published since that time. This more recent work will probably require a re-evaluation of much of the older studies which were done on the so-called "alkaline" FDPase. In the course of older purification procedures, (see reviews cited), FDPase, which in the crude extract was highly active at neutral pH, was extracted in a form with maximal activity above pH 9.0. Until recently, all the molecular studies were performed on this "alkaline" form of the enzyme. In 1971, however, two groups of investigators, using new purification procedures, succeeded in isolating FDPases from rabbit and cow liver respectively, whose pH optima closely resembled those of the crude extracts and which could, therefore, represent the native form of the enzymes.

* Abbreviations: FDP: fructose 1,6-diphosphate; FDPase: fructose 1,6-diphosphatase; SDP: seduheptulose 1,7-diphosphate; SDPase: seduheptulose 1,7-diphosphatase.

With regard to its specificity, FDPase from mammalian sources is almost equally active with SDP as substrate and the two activities appear to be inseparable. In contrast, some organisms have two distinct proteins performing the hydrolyses of FDP and SDP, respectively. It would probably be of advantage for the purpose of our project to use an enzyme which can catalyze both reactions.

I. Mammalian FDPases

A. Liver FDPase

- 1) "Alkaline" - see reviews by Pontremoli and Horecker (1970 and 1971).

Specific activity of crystalline preparation = 19.5 micromole FDP/min. mg protein at room temperature, pH 9.1, 1 mM MnCl_2 .

Requires divalent cation: Mn^{+2} or Mg^{+2} .

With Mn^{+2} the enzyme has little or no activity at neutral pH and maximum activity at pH 9.0-9.2. With Mg^{+2} the pH optimum is strongly influenced by the concentration of metal and the presence of chelating agents, e.g. imidazole.

The enzyme hydrolyzes both FDP and SDP, the latter at about 70% of the rate for FDP. The affinity for SDP is, however, considerably lower than that for FDP. A number of other mono- and diphosphates tested are inactive. Some typical kinetic constants are:

$$\begin{aligned}\text{Rabbit liver: } K_m(\text{FDP}) &= 4.3 \times 10^{-6} \text{ M (pH=9.1, } \text{Mg}^{+2}\text{)} \\ &= \text{less than } 10^{-6} \text{ M (pH=7.5, } \text{Mg}^{+2}\text{)} \\ &= 2.6 \times 10^{-6} \text{ M (pH=9.1, } \text{Mn}^{+2}\text{)} \\ &= \text{less than } 10^{-6} \text{ M (pH=7.5, } \text{Mn}^{+2}\text{)}\end{aligned}$$

(Pontremoli, 1966).

Rat liver: $K_m(\text{FDP}) = 1.2 \times 10^{-5}$ (pH=9.0)

$K_m(\text{SDP}) = 3 \times 10^{-4}$ (pH=9.0)

(Bonsignore et al., 1963).

At higher FDP concentrations, the substrate becomes inhibitory. Thus, at 10^{-3} M FDP the rate of hydrolysis is about 50% of the optimum rate. This substrate inhibition is observed only in the neutral pH range.

FDPases are typically inhibited by AMP, a phenomenon which is assumed to play a role in the physiological regulation of gluconeogenesis; the ratio of AMP/ATP is thought to regulate the relative rate of glycolysis and gluconeogenesis via their effect on phosphofructokinase and FDPase, respectively. Again, the inhibition by AMP is apparent mostly in the neutral pH range.

$K_i(\text{AMP})$ (rat liver) = 1.1×10^{-4} at pH 7.3.

AMP is a noncompetitive inhibitor and is believed to act at an allosteric site. It has been shown for the liver enzyme that the lowered inhibitory effect above pH 9.0 is not due to a lower affinity of the enzyme for the inhibitor. The enzyme can be desensitized to the AMP inhibitor by a number of treatments, without loss of catalytic activity. These very interesting studies, involving selective modification of the protein by various reagents, are reviewed by Pontremoli and Horecker (1970 and 1971) and should be useful in future insolubilization and stabilization studies. Also of interest should be the studies pertaining to activation of the enzyme by disulfide reagents; these increase the activity of the enzyme in the neutral pH range. Thus, in the presence of homocystine the specific activity at pH 7.5 is 40 units/mg protein,

or twice that at alkaline pH, under normal conditions.

With regard to stability, Pontremoli (1966) states that the enzyme can be stored for months in the cold without loss of activity.

The rabbit liver "alkaline" enzyme has been estimated to have a molecular weight of about 130,000 and is made out of a total of four subunits with two types of polypeptide chains of molecular weights about 29,000 and 36,000 respectively. In support of the physical dissociation experiments, various ligands have been found to have four binding sites per molecule of protein.

2) "Neutral"

Traniello et al. (1971b) and Byrne et al. (1971) have succeeded in purifying from rabbit and bovine liver, respectively, FDPases with optimal activity at neutral pH, probably representing the native forms of the enzymes. In view of these studies, it now appears that previous purification procedures led to a proteolytic modification of the protein, which was responsible for the shift from a neutral pH optimum in the crude extract to the alkaline optimum in the pure preparation.

The "neutral" rabbit liver FDPase (Traniello et al., 1971) has a molecular weight of 143,000 (vs. 130,000 for the "alkaline") and a pH optimum of 7.0 - 7.5 with both magnesium and manganese ions.

$$\begin{aligned} K_m \text{ (FDP)} &= 2 \times 10^{-6} \text{ M (pH=7.5, with Mg}^{+2}\text{)} \\ &= 5 \times 10^{-5} \text{ M (pH=9.2, with Mg}^{+2}\text{)} \end{aligned}$$

$$\begin{aligned} \text{Specific activity} &= 14.6 \text{ micromole FDP/min.mg protein} \\ &\quad (\text{Mg}^{+2}, \text{ pH=7.5}) \end{aligned}$$

0.4 mM FDP inhibits the enzyme activity about 50%. This effect is observed only in the neutral pH range.

The cation requirement for "neutral" FDPase is 1-2 mM Mg^{+2} or 0.1-0.2 mM Mn^{+2} . At higher concentrations both cations become inhibitory by 20-30%.

Neutral FDPase is more sensitive to inhibition by AMP than alkaline FDPase. 10^{-4} M AMP is totally inhibitory.

In the case of the bovine hepatic FDPase, Byrne et al. (1971) succeeded in keeping the ratio of activities at pH 6.5 and 9.0 nearly constant throughout the purification procedure. They obtained a preparation, homogenous by several criteria, of specific activity 200 "units"/mg protein at pH=6.5 and 130 "units"/mg protein at pH=9.0, where a "unit" is defined as the amount of enzyme catalyzing the liberation of 1.0 micromole P_i in 30 minutes at 37°. The molecular weight of this protein was about 130,000.

B. Kidney FDPase

May be the same protein as the liver FDPase (see Pontremoli and Horecker, 1971).

C. Muscle FDPase (rabbit)

Acts on both FDP and SDP. Is very sensitive to AMP inhibition. Thus, 1.3×10^{-7} M AMP inhibits the activity by 50% at pH 7.5. SDP hydrolysis is ten times less sensitive to inhibition by AMP.

The maximum rate of FDP hydrolysis at pH 7.5 is 2.5×10^{-5} M. Higher concentrations of FDP are inhibitory. The affinity is lower at pH 9.2. For SDP $K_m = 0.1$ mM at pH 7.5
= 1 mM at pH 9.3

The molecular weight is 133,000.

II. FDPase from Candida utilis

This is an example of a specific FDPase: it does not catalyze the hydrolysis of SDP. The latter function is performed by a separate enzyme whose properties will be discussed below.

A new purification procedure for the Candida FDPase was recently described by Traniello et al. (1971 a). The properties of this preparation are summarized below. An older preparation (Rosen et al., 1966) appears to have fairly similar characteristics.

Molecular weight = 130,000. Tetramer.

Specific activity = 73 micromoles F6P formed/min. mg protein
(Mg^{+2} , 22°, pH = ?).

Maximum activity of crude extracts at pH 8.3 - 8.6.

Maximum activity of purified enzyme at pH 8.6 - 8.8.

EDTA increases activity but does not change pH profile.

K_m (FDP) = 0.8×10^{-5} M at either pH 7.5 or 9.2 (Mg^{+2} and 0.1 mM EDTA).

Substrate concentrations above 0.1 mM are inhibitory.

Inhibited by AMP and activated by disulfide reagents.

The enzyme solution is stable for several months at -20°.

III. SDPase from Candida utilis

(Traniello et al., 1971 a).

Molecular weight = 75,000. Two subunits.

Specific activity = 11.5 micromoles P_i released/min.mg protein
(pH 6.0, 37°).

pH optimum = 6.0 (both in crude extract and purified preparation).

K_m (SDP) = 1 mM; higher concentrations of SDP not inhibitory.

AMP not inhibitory at 3 mM. Metal ion not required. Not modified

by disulfide reagents. Inactive with a number of other phosphates tested, including FDP.

IV. FDPases from Other Microorganisms

A number of other FDPases are reviewed by Pontremoli and Horecker (1971). More recently, a report by Yoshida and Oshima (1971) described the partial purification of the enzyme from a thermophilic bacterium. This enzyme maintains its allosteric properties at 70° and is otherwise similar to other FDPases with alkaline pH optimum.

V. Plant FDPases

Plants appear to have both an "alkaline" and a "neutral" FDPase (see review by Pontremoli and Horecker, 1971) and the "alkaline" enzyme is thought to be the one participating in photosynthesis. In view, however, of the accumulating evidence indicative of the dependence of the pH optimum of FDPase on extraction conditions, it is hard to evaluate what the real situation might be in the living plant, especially as Preiss et al. (1967) have shown that the pH optimum of the so-called "alkaline" enzyme from spinach leaves is strongly dependent on Mg^{+2} concentration. Thus, at 5 mM $MgCl_2$, the pH optimum was about 8.5 and negligible activity was observed at pH 7.0, but when the $MgCl_2$ concentration was raised to 40 mM, the pH optimum shifted to 7.5 and 40% of this activity was found even at pH 7.0.

As an example of a plant enzyme, the FDPase from Euglena gracilis (App, 1966) has a pH optimum of 8.25 $K_m(FDP) = 3 \times 10^{-4}$ M (at a Mg^{+2}/FDP ratio of 20-25); Specific activity = 72 - 128 micromole P_i liberated/min.mg protein. It can be stored for months at -20°, pH 4.95, citrate buffer.

The question of whether the FDPase and SDPase functions in photosynthesis are performed by the same protein appears to be still unresolved.

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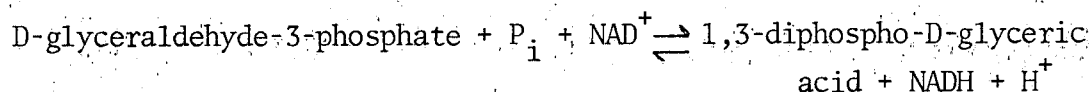
GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

(Reaction 17)

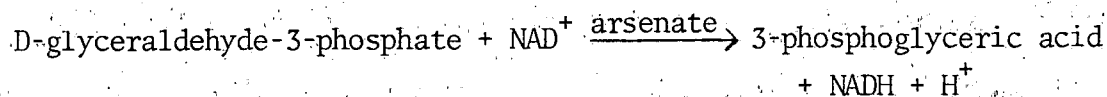
I... The Glycolytic Enzyme

Glyceraldehyde-3-phosphate dehydrogenase (GPD) has been extensively studied but mostly as the enzyme participating in glycolysis: D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating), E.C. 1.2.1.12.

This enzyme catalyzes the reaction:



or, if phosphate is replaced by arsenate;



A comprehensive review about this enzyme is that of Velick and Furfine (1963). The following is a brief summary of the available data:

GPD from a number of sources has been obtained in crystalline form.

Typical preparative procedures are described by Allison (1966).

Molecular weight estimates for the enzyme from various sources are in the range of 120,000 to 140,000.

The enzyme is relatively unstable in solution but quite stable if stored as crystals in the ammonium sulfate mother liquor, in the cold. The stability is improved by the presence of EDTA, β -mercaptoethanol and by the addition of NAD (even though the crystals contain already variable amounts of strongly complexed NAD).

The kinetic constants are difficult to obtain because of their low values and because of product inhibition. For the "reverse" reaction, i.e., for the reduction of P-3PGA, Furfine and Velick (1965) determined the

following values, at pH 7.4, 26°, for the enzyme from rabbit muscle:

Substrates: NADH $K_m = 3.3 \times 10^{-6}$ M

P-3PGA $K_m = 0.8 \times 10^{-6}$ M

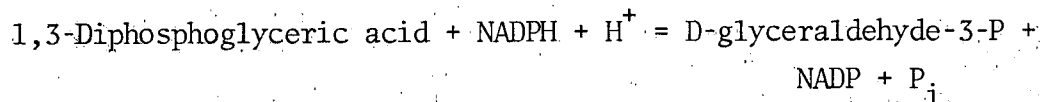
Inhibitors: NAD $K_i = 1.0 \times 10^{-4}$ M

GAld3P $K_i = 6.0 \times 10^{-8}$ M

V_{max} for the reaction in the direction of reduction, at pH = 7.4 is given as 14,900 min⁻¹ (presumably meaning moles of P-3PGA converted per mole enzyme per minute). The same authors calculate V_{max} in the oxidation reaction to be 22,000 min⁻¹, at pH 8.6. However, Allison and Kaplan (1964) give the turnover number for their crystalline enzymes from a variety of sources, at pH 8.5, as 2300 moles NAD reduced per mole enzyme per min. The source of the discrepancy between these numbers is not clear.

II. The Plant Enzymes

In the green plant, the enzyme participating in the photosynthetic sequence is assumed to catalyze the following reaction:



This is the enzyme: D-glyceraldehyde-3-phosphate: NADP oxidoreductase (phosphorylating) - E.C. 1.2.1.13.

Green plants also possess an NAD-linked activity, present in nonphotosynthetic tissues. Also, according to some researchers (see Hageman and Arnon, 1955) a second NAD-linked activity exists in photosynthetic tissues. More recent reports, however, indicate that the NAD- and NADP-linked activities of green tissues may not represent two distinct proteins. (See Schulman and Gibbs, 1968; Melandri et al., 1968).

Schulman and Gibbs (1968) purified the NAD-linked activity of pea seeds and the NAD- and NADP-linked activities of pea shoots and found the following kinetic constants, in the direction of reduction of P-3PGA, at pH 8.5:

| | | | |
|--------------------------|--------|------|---------------------------------------|
| Pea seed (NAD-linked): | P-3PGA | ---- | $K_m = 4.45 \times 10^{-6} \text{ M}$ |
| | NADH | ---- | $K_m = 3.2 \times 10^{-6} \text{ M}$ |
| Pea shoot (NAD-linked): | P-3PGA | ---- | $K_m = 1.57 \times 10^{-6} \text{ M}$ |
| | NADH | ---- | $K_m = 8.0 \times 10^{-6} \text{ M}$ |
| Pea shoot (NADP-linked): | P-3PGA | ---- | $K_m = 1.14 \times 10^{-6} \text{ M}$ |
| | NADPH | ---- | $K_m = 4.0 \times 10^{-6} \text{ M}$ |

The V_{\max} values are not given.

The low Michaelis constants are quite similar to those of the NAD-linked muscle enzymes. Also, in accordance to the finding in the muscle enzyme, the NADP enzyme contains strongly bound nucleotide. Sedoheptulose-7-phosphate and sedoheptulose 1,7-diphosphate were found to inhibit competitively the oxidation of G-3-P by both plant enzymes. The inhibition constants for sedulose diphosphate are:

| | |
|--------------------------|-------------------------|
| Pea seed (NAD-linked): | $K_i = 2.08 \text{ mM}$ |
| Pea shoot (NAD-linked): | $K_i = 1.31 \text{ mM}$ |
| Pea shoot (NADP-linked): | $K_i = 0.6 \text{ mM}$ |

In a recent abstract, McGowan and Gibbs (1971) state that the molecular weight of the chloroplast enzyme is 150,000.

Since the various properties (size, kinetic constants) of the glycolytic (NAD-linked) and photosynthetic (probably NADP-linked) enzymes are very similar, it appears that either would be equally useful for an industrial process, unless one enzyme proves to be more easily insolubilized and stabilized than another. However, if the system is to use a ferredoxin-

linked process for regeneration of reduced pyridine nucleotide, as is done in the chloroplast, then the use of a NADP enzyme would be advantageous, since the K_m for ferredoxin reductase is 400 times lower for NADP than for NAD (Shin and Arnon, 1965).

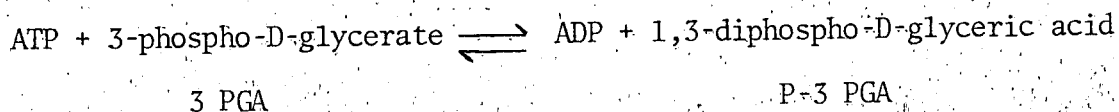
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PHOSPHOGLYCERATE KINASE

(ATP: 3-phospho-D-glycerate 1-Phosphotransferase; E.C. 2.7.2.3)

(Reaction 16)



In spite of the importance of phosphoglycerate kinase (PGK) in both glycolysis/fermentation and photosynthesis, not too much is known about the enzyme at the molecular level. Only the enzyme from yeast appears to have been obtained in highly purified, crystalline form (Bücher, 1955). Axelrod and Bandurski (1953) and Rao and Oesper (1961) have described partial purification of the enzyme from pea seeds and rabbit muscle, respectively.

Larsson-Raźnikiewicz and Malmström (1961) estimated the molecular weight of the yeast enzyme to be 34,000. The enzyme appears to be fairly stable (cf. review by Malmström and Larsson-Raźnikiewicz, 1961). Mg^{+2} is necessary for enzyme activity but Mn^{+2} can substitute for Mg^{+2} to a certain extent.

In their review Malmström and Larsson-Raźnikiewicz (1961) point out a number of reasons why true kinetic constants for PGK are difficult to estimate and that, consequently, the numbers appearing in the literature are not too reliable. The available data for the reaction in the direction of the phosphorylation of phosphoglyceric acid are summarized in Table I. The turnover number for the enzyme catalyzing the reaction in this direction was estimated by Bücher (1955) to be $36,000 \text{ moles/min} \times 10^5 \text{ g enzyme}$; which for a molecular weight of 34,000 means 12,240 moles PGA

converted/min x mole enzyme.

$$K_{eq} = \frac{[ADP][P3PGA]}{[ATP][PGA]} = 3.2 \times 10^{-4} \quad (\text{Bücher, 1955})$$

(at pH 6.9, 25°)

According to the data of Axelrod and Bandurski (1953) the enzyme from pea seed appears to have a broad pH optimum between about 6.8 - 10.

TABLE I: Michaelis Constants for the PGK-catalyzed Phosphorylation of PGA

| Enzyme | pH | Temp. | K_m (moles/l) | | | Reference |
|----------|-----|-------|--|----------------------|--|---|
| | | | Phosphoglycerate | ATP | Mg-ATP ² | |
| Pea seed | 7.3 | 25° | 7.6×10^{-3} | 4.1×10^{-3} | - | Axelrod & Bandurski (1953) |
| Yeast | 6.9 | 25° | 2×10^{-4} | 1.1×10^{-4} | 2.5×10^{-4} | Blücher (1955) |
| Yeast | 7.0 | 38° | - | 1.4×10^{-3} | - | Larsson-Raźnikiewicz & Malmström (1961) |
| Yeast | 7.8 | 25° | - | - | 4×10^{-4} | Larsson-Raźnikiewicz (1964) |
| Yeast | 7.8 | 25° | $6.2-12.3 \times 10^{-4}$ (dep. on [ATP]) | - | $4-10 \times 10^{-4}$ (dep. on [PGA]) | Larsson-Raźnikiewicz (1967) |
| Yeast | 6.0 | 37° | 8.5×10^{-4} | - | - | Rao & Oesper (1961) |
| | 7.0 | 37° | 13.0×10^{-4} | 3.2×10^{-4} | 2.8×10^{-4} | |
| | 8.0 | 37° | 10.7×10^{-4} | 3.3×10^{-4} | - | |
| Rabbit | 6.0 | 37° | 13.6×10^{-4} | - | - | Rao & Oesper (1961) |
| muscle | 7.0 | 37° | 12.2×10^{-4} | 4.8×10^{-4} | 2.7×10^{-4} | |
| | 8.0 | 37° | 10.7×10^{-4} | 4.8×10^{-4} | - | |

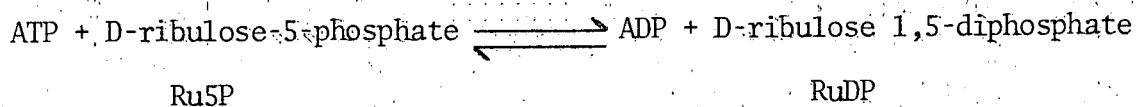
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PHOSPHORIBULOKINASE

(ATP; D-ribulose-5-phosphate 1-phosphotransferase; E.C. 2.7.1.19)

(Reaction 34)



Phosphoribulokinase, like ribulose diphosphate carboxylase, is an enzyme specific to the Calvin cycle. It has not been very extensively investigated.

Phosphoribulokinase has been purified from spinach by Racker (1957) and by Hurwitz et al. (1956). (See also Hurwitz, 1962.) If the specific activities of the two preparations are expressed in the same units, then the value for Racker's enzyme is 90 micromoles RuDP formed/mg protein.min vs. 15.5 micromoles/mg.min for the preparation of Hurwitz et al. (1956). However, since the assays used were different, the values are probably not directly comparable. There is no doubt, though, that Racker's crystalline preparation is the purer of the two.

Properties:

pH optimum: 7.9;

Sensitive to reagents modifying -SH groups; should be stored in the presence of -SH compounds.

$K_m(\text{R5P}) = 2.2 \times 10^{-4} \text{ M};$

$K_m(\text{ATP}) = 2.1 \times 10^{-4} \text{ M}.$

Required a divalent cation. Mg^{+2} is the most effective and shows maximum activity at $5 \times 10^{-3} \text{ M}.$

More recent studies on phosphoribulokinase are concerned mainly with its possible regulatory role (see review by Preiss and Kosuge, 1970).

There is still some disagreement as to whether phosphoribulokinase and carboxydismutase occur as a multienzyme complex (see MacElroy et al., 1968a and Gibson and Hart, 1969).

For the enzyme from Chromatium, Gibson and Hart (1969) determined an approximate molecular weight of 240,000.

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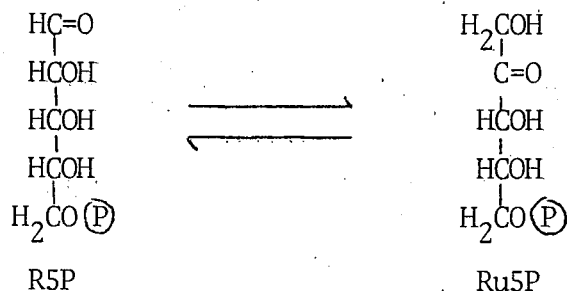
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RIBOSEPHOSPHATE ISOMERASE

(D-Ribose 5-phosphate ketol-isomerase - E.C. 5.3.1.6)

(Reaction 33)

Ribose-5-phosphate isomerase catalyzes the interconversion of ribose-5-P and ribulose-5-P:



The enzyme participates in both autotrophic and heterotrophic pentose metabolism. The isomerase has been purified to various degrees from a number of sources, but there have been rather few careful studies of the protein at the molecular level. Probably the most thorough study of this kind to date is that of Rutner (1970) on the spinach enzyme. An interesting question concerning this enzyme in plants is whether there are one or two species present. Rutner (1970) obtained no evidence of more than one protein in spinach. In contrast, Anderson (1971) reports the existence of two ribose isomerase species in pea leaf - one cytoplasmic, one from the chloroplast. The enzymes are very similar in all properties examined except their isoelectric points which differ by 0.2 pH units. From a metabolic point of view, the observation of the existence of two ribosephosphate isomerases in the heterotroph, *E. coli*, (David & Weismeyer, 1970) is also interesting.

Table I summarizes the available data for the isomerase and its reaction. Where two numbers appear for the specific activity of the enzyme, the one in parenthesis was recalculated on the basis of Rutner's (1970) data for molecular weight and absorption coefficient to make them more easily comparable.

There have been almost no studies involving the chemical modification of the protein and there is little said in the available literature about its stability.

TABLE I: Properties of Ribose 5-P Isomerase from Various Sources

| Source | Mol. wt. | K_m (R5P) (M) | Conditions pH Temp. | Other Observations | Reference |
|--|----------|----------------------|------------------------|---|--|
| Alfalfa | - | - | - | Turnover no. = 240,000 moles Ru5P formed/min. 100,000 g protein (1.25 x 10 moles/min. mole protein) pH optimum = 7.0 $K_{eq} = 0.323$ at 37°; $= 0.264$ at 25.5° $= 0.164$ at 0° Enzyme also present in spinach, orange flavedo, avocado fruit and leaf. | Axelrod & Jang (1954); Axelrod (1955) |
| Spinach | - | - | - | $K = 0.43$ at 38° Enzyme purified to activity of 3000 micromole/min. mg protein | Hurwitz et al. (1956) |
| Erythrocytes | - | 2.2×10^{-3} | 7.5 | pH optimum = 7.5 Probably an SH- protein. | Bruns et al. (1958) |
| <u>Pediococcus</u> <u>pentosaceus</u> | - | 2.8×10^{-3} | 7.6 37° | Protein only partially purified. $K = 0.30$ at 37° | Dobrogosz & DeMoss (1963) |
| <u>Aerobacter</u> <u>aerogenes</u> | - | 1.8×10^{-3} | 8.0 30° | Protein 11 times purified. Most active at pH 8.5 & 30° Most stable at pH 8.0 Temp. optimum = 30° Probably an SH- protein. | Matsushima & Simpson (1965) |
| <u>Rhodospirillum</u> <u>rubrum</u> | 57,000 | 4×10^{-3} | 7.0 37° | pH optimum = 7.0 - 7.4 A number of inhibitors discussed in relation with the possible regulatory role of the enzyme. K_i (AMP) = 1.2 ± 0.2 mM K_i (P.) = 2.4 ± 9 mM K_i (RuDP) = 0.033 ± 0.005 mM (The isomerase has a higher apparent affinity for RuDP than RuDP carboxylase!) | Anderson et al. (1968) Anderson & Fuller (1969) |

TABLE I (Continued)

| Source | Mol. wt. | K_m (R5P) (M) | Conditions pH Temp. | Other Observations | Reference |
|--|----------|--|------------------------|--|--------------------------|
| Spinach | - | 4.6×10^{-3} | 7.3 37° | Activity: 5000 micromoles/min. "mg" protein (Protein determined spectro- photometrically) (1.13×10^5 mole/min.mole enzyme) | Knowles et al. (1969) |
| <u>E. coli</u> (two enzymes) | - | $1.85-2.59 \times 10^{-3}$ $0.13-0.25 \times 10^{-3}$ | - | Heat stable Heat labile | David & Wiesmayer (1970) |
| Spinach | 53,000 | 4.6×10^{-4} | 7.1 37° | Purity checked in the ultra- centrifuge & by gel electro- phoresis. Activity: 1.21×10^5 mole/min. mole enzyme | Rutner (1970) |
| Pea leaf chloroplast cytoplasmic | - | 2.3×10^{-3} 2.1×10^{-3} | 7.5 25° | Isoelectric points: 5.0 4.8 Both enzymes have broad pH curve centered at 7.5. K_i (AMP) = $1 - 13 \times 10^{-3}$ M (for both) K_i (P _i) = about 10 mM RuDP increases V_{max} of both. | Anderson (1971) |

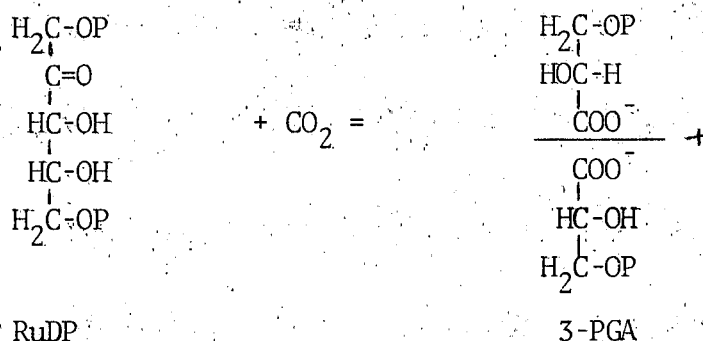
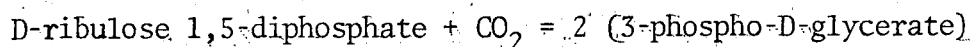
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RIBULOSE DIPHOSPHATE CARBOXYLASE

(3-Phospho-D-glycerate carboxy-lyase (dimerizing); E.C. 4.1.1.39)

(Reaction 15)



I. The Enzyme from Higher Plants

Although RuDP carboxylase is present in all autotrophic and chemotrophic organisms, it has been most extensively studied in higher plants. Here, it is present as a component of high sedimentation coefficient (about 18S) constituting some 50% of the soluble leaf protein. For some time this protein had been isolated and purified without its enzymatic role being recognized, and it was known only as "Fraction I Protein". Later it became apparent, and now it is almost universally recognized, that "Fraction I Protein" is the RuDP carboxylase of plants.

As can be seen from the studies summarized in Table I, the molecular weight estimates for the plant enzyme range between 4.8 and 5.6×10^5 . The references given describe suitable purification procedures.

TABLE I. Molecular Weights of RuDP Carboxylase from Higher Plants

| Organism | Molecular Weight $\times 10^{-5}$ (by sedimentation equilibrium) | Reference |
|-----------------|---|---------------------------|
| Spinach | 5.15 | Trown (1965) |
| Spinach | 5.57 | Paulsen & Lane (1966) |
| Spinach | 4.75 | Pon (1967) |
| Spinach beet | 5.61 | Ridley et al. (1967) |
| Chinese cabbage | 5.11 | Kieras & Haselkorn (1968) |

Carboxylase preparations have been obtained in highly purified state, as evidenced by chromatographic, electrophoretic, and ultracentrifugal analyses. Recently, however, Kawashima and Wildman (1971) have succeeded in crystallizing the enzyme from tobacco leaves. The preparation, which appeared pure by other criteria, nevertheless showed a higher specific activity after crystallization. The authors suggested that some molecules in the mother liquor had undergone a configurational change which had led to both their inactivation and their inability to crystallize.

The enzyme activity is highly dependent on magnesium ions. Reports in the literature disagree as to whether other cations can replace Mg^{+2} . Variation in magnesium concentration leads to shifts in pH optima as well as Michaelis constants, as evidenced by the data compiled in Tables II and III.

TABLE II. Variation of the pH Optimum of RuDP Carboxylase with Magnesium Ion Concentration

| Organism | Mg^{+2} (mM) | pH Optimum | Reference |
|----------|----------------|------------|---------------------------------------|
| Spinach | 1.8 | 8.5 | Bassham et al. (1968) |
| | 45 | 7.7 | " |
| Spinach | 0 | 8.5 | Sugiyama, Nakayama & Akazawa (1968-b) |
| | 1 | 7.5 | " |
| | 10 | 6.5 | " |

TABLE III. Kinetic Data for RuDP Carboxylase from Higher Plants

| Organism | Temp. | pH | Mg ⁺² (mM) | K _m (RuDP) | | K _m (HCO ₃ ⁻) | | Reference |
|--------------------|-------|-----|--------------------------|-------------------------|--|---|-----------------------|------------------------------|
| | | | | K _m | Conc. of HCO ₃ ⁻ (mM) | K _m | Conc. of RuDP (mM) | |
| Spinach | 25 | 7.7 | 9.3 | 2.5 x 10 ⁻⁴ | 46 | 1.1 x 10 ⁻² | 1.0 | Weissbach et al. (1956) |
| Spinach | 25 | 7.8 | 10 | | | 2 x 10 ⁻² | 3.7 | Racker (1957) |
| Spinach | 30 | 7.8 | 10 | 1.2 x 10 ⁻⁴ | 50 | 2.2 x 10 ⁻² | 0.7 | Paulsen & Lane (1966) |
| Chinese cabbage | 25 | 8.0 | 10 | 1.0 x 10 ⁻⁴ | 50 | 3 x 10 ⁻² | 0.7 | Kieras & Haselkorn (1968) |
| Spinach | 25 | 8.5 | 0 | | | 2 x 10 ⁻² | 0.7 | Sugiyama et al. (1968b) |
| | | | 5 | | | 1.2 x 10 ⁻² | 0.7 | " |
| | | | 20 | | | 5.6 x 10 ⁻³ | 0.7 | " |
| Spinach | 23 | 7.7 | 1.8 | | | 5.4 x 10 ⁻³ | 0.136 | Bassham et al. (1968) |
| | | | 45.4 | 2.5 x 10 ⁻⁴ | 0.33 | 2.5 x 10 ⁻³ | 0.136 | " |
| Spinach | 25 | 7.5 | 10 | 7 x 10 ⁻⁴ | 50 | | | Sugiyama et al. (1969) |
| Barley | 28 | 8.0 | 30 | 1.45 x 10 ⁻⁴ | 60 | 2.2 x 10 ⁻³ | 0.5 | Kleinkopf et al. (1970) |

As can be seen from Table III, the enzyme has a rather high affinity for RuDP but a very low one for HCO_3^- . Even at saturating bicarbonate concentration, V is only 1340 moles CO_2 fixed/min x mole enzyme at 30° (Paulsen and Lane, 1966). Since in the living plant the concentration of CO_2 is even smaller, this feature of the enzyme has posed a dilemma as to how it can function efficiently in vivo. Recently, there has been an indication (Cooper et al., 1969) that perhaps the real substrate for the enzyme is not HCO_3^- but molecular CO_2 , which would mean that the real K_m is about 50 times lower.

RuDP carboxylase appears to be highly specific: ribulose 5-phosphate, ribose 1,5-diphosphate and other compounds tested (Weissbach et al., 1956) could not replace RuDP.

Phosphate and sulfate ions and PGA are inhibitory with K_i 's of about 4 mM, 8 mM and 8-9 mM, respectively (see Paulsen and Lane, 1966). RuDP becomes inhibitory at concentrations higher than 0.7 mM.

Reagents that attack SH groups are strong inhibitors of the enzyme. A number of studies have addressed themselves to the question of whether SH groups are directly involved in the binding of RuDP to the enzyme or cause a generally unfavorable configurational change and, although the results are not entirely clear, this work might be useful to the researchers attempting to achieve insolubilization of the enzyme: Rabin and Trown (1964); Sugiyama et al. (1967, 1968a,d); Akazawa et al. (1968).

Ogren and Bowes (1971) have shown that oxygen inhibits the carboxylase from soybean and in another report these authors (Bowes and Ogren, 1971) indicated that the enzyme is also inhibited by a number of nucleotides and sugar phosphates, most notably fructose diphosphate, ATP, and NADPH. Non-phosphorylated sugars were not inhibitory.

As far as the stability of the enzyme is concerned, Trown (1965) has shown that reduced glutathione, 1.0 M sucrose, and 0.05% Triton (a non-ionic detergent) protected the enzyme from spinach against deactivation. In contrast, the ionic detergent sodium dodecyl sulfate rapidly destroyed its activity. Kawashima et al. (1971), using a crystallized preparation of the tobacco enzyme, made the interesting observation that, while the specific activity of the enzyme did not change during 12 days at room temperature, if the enzyme was stored at 0° it lost 70% of its activity in one day; the activity of the cooled preparation could be restored by heating for 20 minutes at 50°C. That the enzyme is quite heat-stable is also indicated by the fact that one of the standard purification procedures (Racker, 1962) involves heating the crude preparation at 80°, then 60°. Racker (1962) also recommends a protamine-chloroform treatment during extraction of the enzyme purported to increase its stability and stresses the fact that the enzyme is unstable below pH 6 and that, therefore, pH's below neutrality should be avoided during purification.

Two other important areas of research concerning the plant carboxylase should be mentioned, although a more detailed description of the findings would be beyond the scope of the present summary. These areas are:

- 1) Elucidation of the subunit structure of the protein. The reader is referred to the following papers: Rutner and Lane (1967), Sugiyama and Akazawa (1967 and 1970), Sugiyama, Matsumoto and Akazawa (1970) and Moon and Thompson (1971).
- 2) Studies on the actual mechanism of the enzyme; Pon et al. (1963), Akoyunoglou and Calvin (1963), Trown and Rabin (1964), Mullhofer and Rose (1965), Wishnick et al. (1969), Cooper et al. (1969).

II. The Enzyme from Microorganisms

There have been a number of investigations of the RuDP carboxylase from organisms other than higher plants but none as thorough as some of the work summarized above on the plant enzyme. The findings in some of these studies are summarized in Table IV. There appear to be a number of discrepancies in the data, especially in the papers from Akazawa's group, which disagree not only with the findings of others but also within their own consecutive papers.

Perhaps the one point worth stressing is the lower molecular weight of the enzyme from the Athiorodaceae, especially Rhodospirillum rubrum. As the kinetic constants of this enzyme are approximately the same as that of the plant enzyme, the smaller molecular weight might present an advantage for a possible industrial process.

TABLE IV. Some Properties of Ribulose Diphosphate Carboxylase from Microorganisms

| | Sed. Mol. Wt. Const. | K_m (HCO_3^-) | K_m (RuDP) | Remarks | Reference |
|--------------------------------|-------------------------|--------------------------|--------------------------|---|--|
| GREEN ALGAE | | | | | |
| <u>Chlamydomonas reinhardi</u> | 16-18 | | | | Kieras & Haselkorn (1968) |
| <u>Chlorella ellipsoidea</u> | 18.4 | 4.7×10^5 | 11.9×10^{-4} | Stimulated by Mg^{+2} | Akazawa et al. (1969) Sugiyama et al. (1969) Matsumoto et al. (1969) |
| <u>Euglena gracilis</u> | 19 | | | Absent in dark | Anderson et al. (1968) |
| BLUE-GREEN ALGAE | | | | | |
| <u>Plectonema boryanum</u> | 16-18 | | | | Kieras & Haselkorn (1968) |
| <u>Anacystis nidulans</u> | 19.5 | | | | Anderson et al. (1968) |
| THIORODACEAE | | | | | |
| <u>Chromatium</u> | 19.7 16-18 | $9 \pm 5 \times 10^{-2}$ | $4 \pm 1 \times 10^{-4}$ | | Anderson et al. (1968) Kieras & Haselkorn (1968) |
| ATHIORODACEAE | | | | | |
| <u>Rps. palustris</u> | 12 | | | | Anderson et al. (1968) |
| <u>Rps. sphaeroides</u> | 14.5 | | | | Anderson et al. (1968) |
| | | | | Stimulated by Mg^{+2} pH optimum not affected by Mg^{+2} | Sugiyama & Akazawa (1968) |
| | | 2.4×10^5 | | | Akazawa et al. (1970) |

| | Sed. Mol. Wt. Const. | K_m (HCO_3^-) | K_m (RuDP) | Remarks | Reference |
|--|-------------------------|---------------------|--|--|--|
| ANTHIORODACEAE (continued) | | | | | |
| <u>R. rubrum</u> | 6.2 | 1.2×10^5 | 0.8×10^{-4} 0.8×10^{-4} | Mg^{+2} not required pH opt. = 8.1 Mg^{+2} required pH optimum not $+2$ affected by Mg | Anderson et al. (1968) Anderson & Fuller (1969) Akazawa et al. (1969) Akazawa et al. (1970) |
| CHEMOTROPHIC BACTERIA | | | | | |
| <u>Hydrogenomonas</u> <u>eutropha</u> | 20 | 5.15×10^5 | 1.25×10^{-4} | Mg^{+2} required | Kuehn & McFadden (1969 a,b) |
| <u>Hydrogenomonas</u> <u>facilis</u> | | 5.51×10^5 | 4.16×10^{-3} 2.35×10^{-4} | Mg^{+2} required | Kuehn & McFadden (1969 a,b) |

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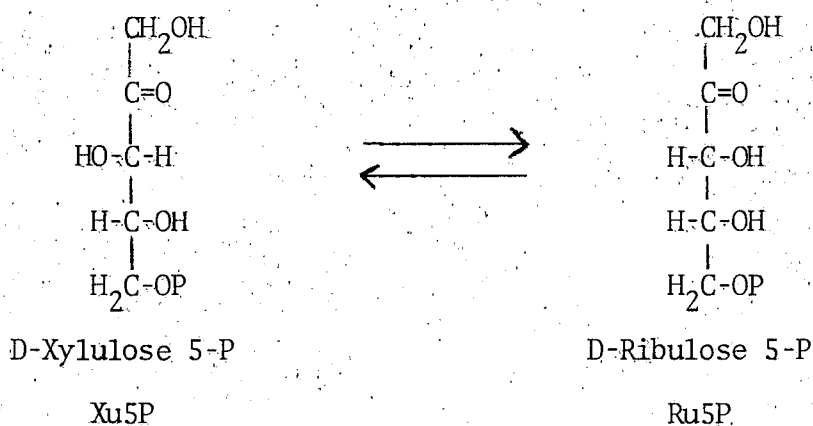
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RIBULOSEPHOSPHATE 3-EPIMERASE

(D-RIBULOSE-5-PHOSPHATE 3-EPIMERASE; E.C. 5.1.3.1)

(Reaction 32)



There are but a limited number of studies available on ribulose phosphate 3-epimerase.

The enzyme was purified about 700 fold from Lactobacillus pentosus by Hurwitz and Horecker (1956). This preparation had a specific activity of 345 "units"/mg, based on an assay coupling the epimerase and isomerase reactions: Xu5P $\xrightarrow{\text{epimerase}}$ Ru5P $\xrightarrow{\text{isomerase}}$ R5P, where 1 unit was defined as the amount of enzyme which formed 1 micromole R5P in 5 minutes (pH=7.5, 25°). The enzyme had a pH optimum between 7.0 and 8.0. The maximum reaction velocity was reached at a Xu5P concentration of 1.6×10^{-3} , and the K_m (Xu5P) was 5.0×10^{-4} M. The equilibrium constant for the reaction $K_{eq} = [\text{Xu5P}]/[\text{Ru5P}] = 1.5$ (at 25°). The enzyme was stable when stored in the cold and was quite specific.

Ru5P 3-epimerase has also been purified from spleen (Ashwell and Hickman, 1957); the properties of this preparation were quite similar to those of the bacterial enzyme, above.

For the yeast enzyme (Williamson and Wood, 1966), the molecular weight was determined to be about 46,000.

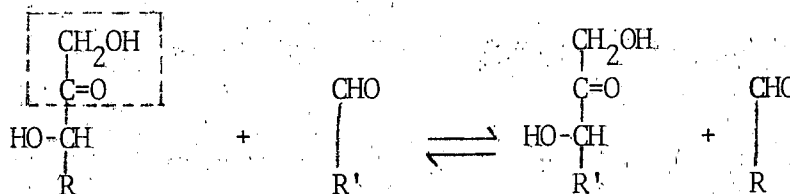
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TRANSKETOLASE

(Sedoheptulose-7-Phosphate; D-Glyceraldehyde-3-Phosphate Glycol Aldehyde
Transferase; E. C. 2.2.1.1)

(Reactions 28, 31)



"donor ketose" "acceptor aldose"

As is the case with a number of enzymes reviewed in the present report, transketolase has been studied mostly as an enzyme participating in a degradative pathway, in this case the pentose phosphate shunt (oxidative pentose phosphate pathway). Therefore the data available once again describe reactions which are the inverse of those taking place in the synthetic, Calvin cycle (reductive pentose phosphate cycle).

Known "donors" for the above transketolase-catalyzed reaction are: xylulose-5-phosphate, fructose-6-phosphate, sedoheptulose-7-phosphate, hydroxypyruvate, L-erythrulose, D-xylulose, octulose-8-phosphate (see review by Racker, 1961); D-ribose, L-lyxose, D-xylose, L-arabinose (Villafranca and Axelrod, 1971). The only requirement appears to be a trans configuration at the 3 and 4 positions. Acceptors include: glyceraldehyde-3-phosphate, D-erythrose-4-phosphate, D-ribose-5-phosphate, formaldehyde (when hydroxypyruvate is donor), glycolaldehyde, glyceraldehyde, D-deoxyribose-5-phosphate, allose-6-phosphate, glucose-6-phosphate and D-arabinose-5-phosphate.

Few data on the protein are available at the molecular level.

Transketolase from yeast has been purified and studied by de la Haba et al. (1955), Srere et al. (1958), Datta and Racker (1961 a and b); see also review by Racker (1961).

The specific activity of the crystalline preparation of Datta and Racker (1961a) was 24 micromoles/min.mg protein, with Xu5P and R5P as substrates, at the optimum pH of 7.6. The equilibrium constants for some of the transketolase reactions, measured at 25°, with the crystalline yeast preparation were: (Datta and Racker, 1961a; Racker, 1961).

| <u>Reaction</u> | <u>K_{eq}</u> |
|---|-----------------------|
| xylulose-5-P + ribose-5-P \rightleftharpoons Sedoheptulose-7-P + glyceraldehyde-3-P | 1.2 |
| xylulose-5-P + erythrose-4-P \rightleftharpoons fructose-6-P + glyceraldehyde-3-P | 10.3 |
| fructose-6-P + glycolaldehyde \rightleftharpoons erythrulose + erythrose-4-P | 0.015 |

The following kinetic constants were measured by Datta and Racker (1961a):

| | | |
|--|---|--------------------------|
| K _m (Xu-5-P) in the presence of R-5-P | = | 2.1 x 10 ⁻⁴ M |
| K _m (F-6-P) in the presence of R-5-P | = | 1.8 x 10 ⁻³ M |
| K _m (Eu-3-P) in the presence of G-3-P | = | 4.9 x 10 ⁻³ M |
| K _m (R-5-P) in the presence of Xu-5-P | = | 4.0 x 10 ⁻⁴ M |

The molecular weight of the yeast preparation was estimated to be 140,000. Recently, Heinrich and Wiss (1971a) reported that yeast transketolase is made of two subunits of molecular weight 70,000. The enzyme from yeast requires Mg⁺² and thiamine pyrophosphate for activity. Thiamine, thiamine monophosphate and thiamine triphosphate cannot replace TPP. Sulfate and phosphate ions are inhibitory at concentrations above 0.01 M.

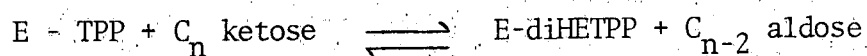
Transketolase has also been purified from human erythrocytes (Heinrich and Wiss, 1971b). The preparation still contained glyceraldehyde-3-P dehydrogenase. Its specific activity was 2.38 micromole S7P formed/min.mg pro-

tein at 37°. The molecular weight was estimated to be 136,000. The K_m measured with the "pentose phosphate mixture" (R-5-P + Ru-5-P + Xu-5-P) was 1.7×10^{-3} M. No cofactor requirement was found. Metal ion does not appear to be required and TPP is probably tightly bound to the enzyme. Sulfate and phosphate ions were inhibitory above 10 mM. The pH optimum was 7.75.

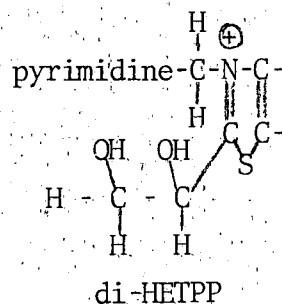
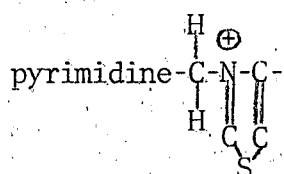
Other sources from which transketolase has been purified include spinach (Bonsignore et al., 1962) and Candida (Kiely et al., 1969).

Studies on the mechanism of action of transketolase, especially with regard to the function of the thiamine pyrophosphate are reviewed by Kram-pitz (1969).

The mechanism involves a two-reaction sequence in which the intermediate is tightly bound to the enzyme. In the first reaction, the keto substrate is cleaved with formation of a two-carbon adduct of thiamine pyrophosphate- α,β -dihydroxyethyl thiamine diphosphate:

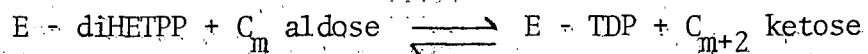


Partial structures:



Both TPP and diHETPP are tightly bound to the enzyme; thus, glyceraldehyde is not detected in the reaction mixture and the detection of glyceraldehyde 3-phosphate and di-HETPP necessitates stoichiometric amounts of enzyme. The two-carbon moiety must be transferred to an acceptor aldose

before the enzyme-TPP complex becomes available for a new reaction sequence:



Since the enzyme is rather nonspecific with respect to the acceptor aldose, it is clear that when a number of compounds are present in the reaction mixture the direction in which the overall reaction will proceed will depend on the relative concentrations of the various compounds and the relative rates of reaction. Any systems design ought to take these factors into consideration, although it is possible that in a steady-state situation, such as we encounter in the operation of a cyclic process, the system will become self-regulatory.

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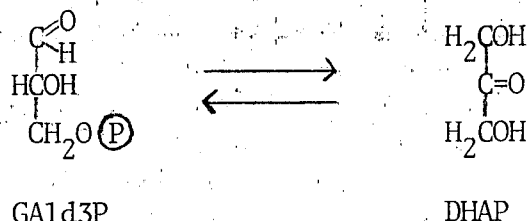
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TRIOSEPHOSPHATE ISOMERASE

(D-Glyceraldehyde-3-phosphate Ketol-isomerase; E.C. 5.3.1.1)

(Reaction 21)

Triosephosphate isomerase catalyzes the reversible conversion of D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate;



The enzyme is present at high levels in both animal and plant tissues. Crystalline preparations of the enzyme have been obtained from calf muscle (see Beisenherz, 1955), yeast (Krietsch et al., 1970), rabbit muscle (Norton et al., 1970), and horse liver (Lee et al., 1971). Purified enzyme has also been prepared from pea seeds (Turner et al., 1965), green algae (Meeks et al., 1968), human liver (Lee et al., 1971) and other sources. Recently, Anderson (1971) has succeeded in separating by isoelectric focusing the cytoplasmic and chloroplast pea leaf enzymes.

Estimates of the enzyme molecular weight and the kinetic parameters of the reaction in the direction of conversion of glyceraldehyde 3-phosphate to dihydroxyacetone are summarized in Table I. The list is probably not exhaustive but the enzymes from various sources appear to have very similar properties. Triosephosphate isomerase is a protein of molecular weight 50,000 - 60,000, probably composed of two similar subunits. The enzyme has a very high activity and the Michaelis constant for glyceraldehyde 3-phosphate is low. The equilibrium constant for the reaction is about 22 ([dihydroxyacetone phosphate]/[D-glyceraldehyde 3-phosphate]).

There have been a number of studies directed at elucidating the mechanism of the triosephosphate isomerase reaction and the active center of the enzyme. (See, for example, Burton & Waley, 1966, 1967 and 1968a; Hartman, 1970, 1971; Wolfenden, 1970; Miller & Waley, 1971). Hartman (1971) has sequenced 15 amino acids around the active center containing an essential glutamyl residue. Since the studies mentioned often involved chemical modification of the protein, they should be of use to the researcher working on the insolubilization of the isomerase. In this regard it should be mentioned that Krietsch et al. (1970) found the yeast enzyme to be very resistant to protein modifying reagents.

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TABLE I. Properties of Triosephosphate Isomerase from Various Sources

| Source | Mol. Wt. | K_m (G-3-P) (M) | Conditions pH Temp. | Other observations | Reference |
|---------------------------|----------|-----------------------|------------------------|--|------------------------------|
| Calf muscle | - | 3.9×10^{-4} | 7.5 26° | Inhibited by phosphate 75% by 0.05 M pH optimum between 7-8 activity decreased by 50% at 6.3; $K_{eq} = 22$ Turnover number = 9.45×10^5 moles G3P converted/min. 10^5 g enzyme at saturating substrate conc. & 26°; twice as high at 38°. | Beisenherz (1955) |
| Pea seed | - | 3.6×10^{-4} | 7.2 | Inhibited by arsenate, cysteine, sulfate, p-chloromercuriben- zoate, chloride Broad pH optimum 7.2 - 8.9 High activity Enzyme present in other plant tissues. | Turner et al. (1965) |
| Ankistrodesmus braunii | - | 4.34×10^{-4} | 7.5 | } Both enzymes have sharp pH optimum at 7.7 Inhibited by phosphate, sulfate. | Meeks et al. (1967) |
| Scenedesmus acuminatus | - | 9.7×10^{-4} | 7.5 | | |
| Rabbit muscle | 60,000 | 4.6×10^{-4} | 7.8 25° | $V_{max} = 6700$ units/mg protein (micrograms G3P converted/ min. mg protein) K_i phosphate = 6×10^{-3} M K_i DL α -glycerophosphate = 2.3×10^{-4} M $K_{eq} = 22$ | Burton & Waley (1968 a,b) |

| Source | Mol. Wt. | K_m (G-3-P) (M) | Conditions | | Other observations | Reference |
|----------------------|-----------|----------------------|------------|-------|---|---------------------------|
| | | | pH | Temp. | | |
| Yeast | 56-60,000 | 1.3×10^{-3} | 7.6 | 25° | Activity: 1.0×10^6 moles G3P/min. mole enzyme. $K_{eq} = 19$ pH optimum about 7-9 K_1 arsenate = 12 mM Two subunits. | Krietsch et al. (1970) |
| Rabbit muscle | 56-60,000 | 3.2×10^{-4} | 7.6 | 25° | pH optimum 6.5 - 10 Activity $5.1-5.2 \times 10^5$ $K_{eq} = 18.9$ K_1 arsenate = 6.2 mM Two subunits. | |
| Rabbit liver | 56-60,000 | 3.2×10^{-4} | 7.6 | 25° | | |
| Rabbit muscle | 53-56,000 | | - | - | Activity = 7500 micromole/ min.mg protein Two subunits of M.W. 26,500. | Norton et al. (1970) |
| Pea leaf chloroplast | - | 4.2×10^{-4} | 7.5 | 25° | pH optimum = 7.8 isoelectric point = 4.75 | Anderson (1961) |
| cytoplasmic | - | 2.0×10^{-4} | 7.5 | 25° | pH optimum = 7.1 isoelectric point = 4.8 $K_{eq} = 24$ at 25° Both enzymes inhibited by phosphate, arsenate, sulfate, & a number of metabolites, most strongly by P-glycollate. | |
| Horse liver | - | - | - | - | Sp. activity = 3183 $V_{max} = 8000$ micromole/min.mg pro- tein (discrepancy unclear) | Lee et al. (1971) |

| Source | Mol. Wt. | K_m (G-3-P) (M) | Conditions | | Other observations | Reference |
|---------------|-----------|----------------------|------------|-------|---|-------------------|
| | | | pH | Temp. | | |
| Human liver | 46-49,000 | - | - | - | Sp. activity = 2397 V_{max} = 2300 micromole/min.mg protein | Lee et al. (1971) |
| Rabbit muscle | 44-49,000 | - | - | - | V_{max} = 7200 micromole/min.mg protein | |

ENZYME INSOLUBILIZATION AND STABILIZATION

The development in recent years of a variety of methods for the insolubilization of proteins has opened for the first time the possibility of large-scale utilization of enzymes in industrial processes. Reactions performed in the presence of insoluble enzyme derivatives are biphasic and the enzymes can easily be recovered by mere filtration and reused as long as they remain active; alternatively, insoluble enzyme preparations can be packed in columns, again eliminating the need for separation. In a majority of the cases reported so far, though by no means in every case, insolubilization of enzymes has resulted in improved enzyme stability, another important factor in a possible industrial application. The reason for this phenomenon appears to be a decreased susceptibility to denaturation and, in the case of proteolytic enzymes, which have constituted a large proportion of the enzymes whose insolubilization has been attempted to date, a decreased amount of self-digestion. The most serious difficulty encountered in the process of insolubilizing enzymes is the relative loss of activity due to the various treatments to which the enzyme is subjected.

One could, therefore, summarize the main requirements for the successful industrial application of an enzymatic process, as follows:

- 1) that the enzyme derivative remain sufficiently active for the process to take place within a reasonable time span;
- 2) that the derivative be truly insoluble, i.e. that no leakage occur during the various stages of the process. This must be tested under the actual conditions of the projected process, as it has been shown that small changes in conditions can bring about desorption of the enzyme; and

3) that the enzyme remain stable for periods long enough to make its use economically feasible. In this regard, one must of course consider the cost of the large-scale preparation of the enzyme itself.

A variety of methods for the preparation of water-insoluble protein derivatives has been developed and an overall outline of these procedures is attached. In general, the procedures fall into two large groups: those involving only a physical immobilization of the protein and those based on a chemical reaction linking the protein molecules either to an insoluble support, or to each other, or both. Each method has its advantages and disadvantages. In the past, most work has been done using enzymes covalently bound to polymeric matrixes. More recently, the use of porous glass as a support for covalently attached enzymes appears to be gaining considerable application.

The choice of a particular method for the insolubilization of a certain enzyme appears to be largely empirical as not enough generalizations have emerged from the cases studied so far. A few possible starting considerations might be listed. Thus, in the case when covalent binding of the protein to a support is envisaged, knowledge of the primary structure around the active site might be useful in determining which modifying agents should be avoided. When the substrate for a reaction is charged, the use of polyionic matrices of the same charge is to be avoided, due to the negative effect of the charge repulsion; conversely, the use of an oppositely charged matrix might be of advantage. Another factor to be considered is the size of the substrate. Thus, in the case of large substrates one should avoid using methods in which the enzyme is physically

occluded in a matrix, as the reaction could be highly hindered by the diffusion of the substrate into the matrix. Generally speaking, however, the procedure adopted would have to be largely a matter of trial-and-error.

A number of reviews on insolubilized enzymes are now available and are listed in the attached bibliography. The recent review by Melrose (1971) (see Section I of bibliography) is particularly up-to-date and well-documented in the various areas of interest both to the biochemist and to the chemical engineer.

We have also compiled a partial bibliography in which the available literature is classified according to the method of insolubilization used.

Two alternatives to the usual methods of immobilizing enzymes by insolubilization could also be mentioned. In a recent paper, Wykes, Dunnill and Lilly (Biochim. Biophys. Acta 250, 522, 1971) reported the immobilization of amylase by attachment to soluble support materials. The enzyme was attached to various polymers, in the usual manner, but the latter were in a soluble form. This permits easy separation by ultrafiltration but removes some of the diffusion and steric barriers encountered by high molecular-weight substrates in the case of water-insoluble supports. Another interesting possibility is that studied from a theoretical point of view by Rony (1971) (see Section II of bibliography) of occluding the enzymes within hollow fibers. This precludes the need for insolubilization and provides at the same time an automatic means of separation of the low molecular-weight reactants. However this method would have no advantage from the point of view of increasing enzyme stability as compared to conventional enzyme reactions in solution.

OUTLINE OF
METHODS FOR PREPARATION OF WATER-INSOLUBLE PROTEIN DERIVATIVES

A. PHYSICAL ADSORPTION ON:

1. Neutral surfaces; glass beads, quartz, charcoal particles, dialysis tubing, Millipore filters, collodion membranes, silica gels;
2. Ion-exchange resins: cationic: DEAE-cellulose and DEAE-Sephadex;
anionic: carboxymethyl (CM)-cellulose.

Disadvantage of the method: variation of pH, ionic strength, temperature or addition of substrate may lead to desorption.

B. OCCLUSION IN CROSSLINKED POLYMERIC MATRICES

(Carry out polymerization reaction in aqueous solution containing enzyme.) Polymer most frequently used: acrylamide + N, N-methylene-bisacrylamide. Gel can then be dispersed into particles.

Advantage: imposes minimal constraint on enzyme, no covalent bond formation, could be applied to any enzyme.

Disadvantages:

- a) because of broad distribution of gel pore size, leakage of enzyme is difficult to avoid;
- b) enzyme reaction occurs within domain of gel matrix, therefore reaction limited to substrates which can diffuse readily into gel;
- c) free radicals generated during polymerization may affect activity of enzyme.

C. COVALENT BINDING TO WATER-INSOLUBLE CARRIERS

Should be done:

- a) via functional groups non-essential for activity;

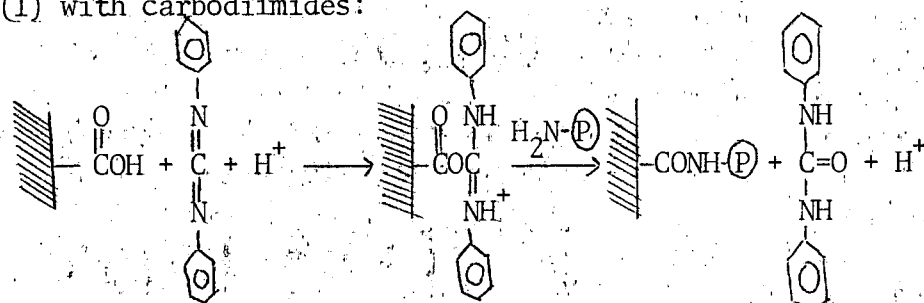
b) under non-denaturing conditions.

Functional groups suitable for covalent binding; α and ϵ amino groups; γ , β and γ carboxyl groups; phenol ring of tyrosine; -SH of cysteine; -OH of serine; imidazole of histidine.

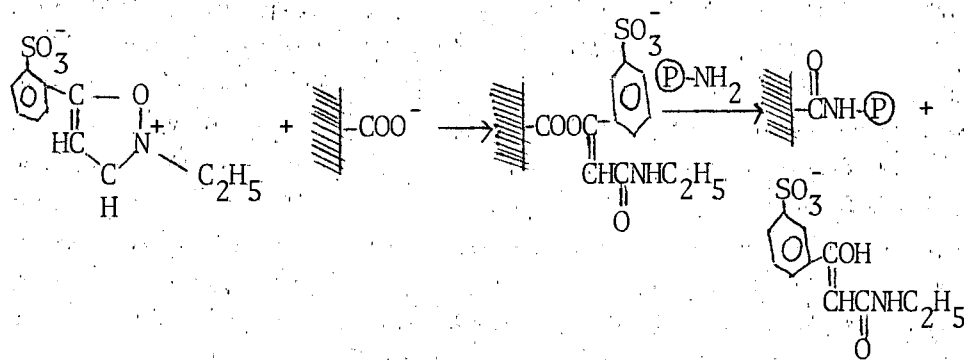
Some methods commonly used:

a) Binding of proteins via amino groups to activated carboxyl matrices. Activation can be achieved:

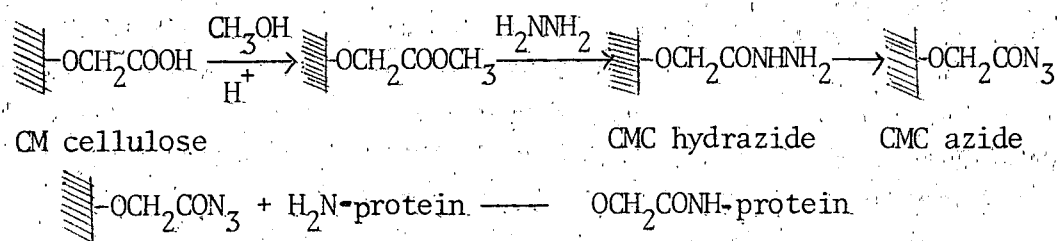
(1) with carbodiimides:



(2) with Woodward's reagent (N-ethyl-5-phenylisoxazolium-3'-sulfonate):

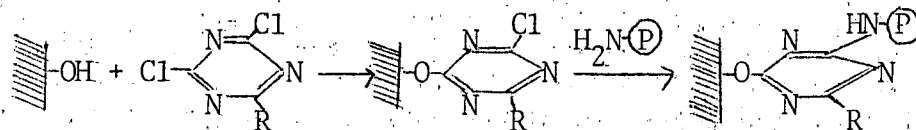


(3) by transformation into the corresponding azides:

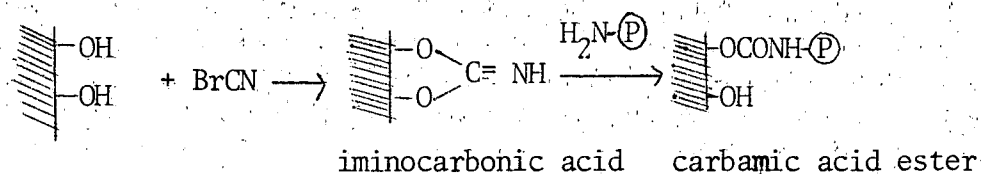


b) Binding of proteins via amino groups to activated hydroxyl matrices.

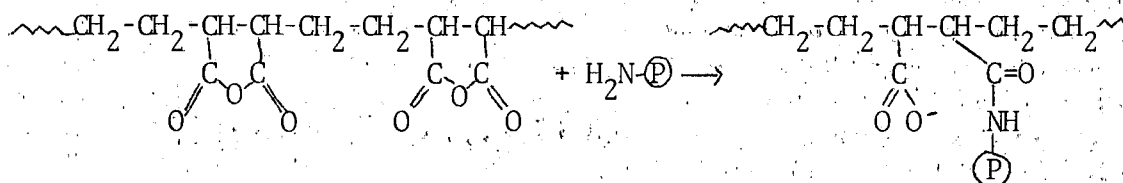
(1) with chloro-s-triazines;



(2) with cyanogen halides:



c) Acylation of protein amino groups by ethylenemaleic acid (EMA) copolymer:

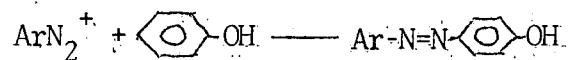
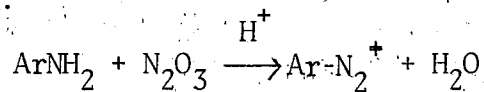


d) Binding to diazotized resins, mainly via tyrosine residues
(other groups reacting are -NH_2 , lysine, arginine, histidine).

A number of different carriers have been used; these possess

-NH₂ groups which are diazotized, then reacted with the pro-

tein:



Some carriers used:

a) Synthetic organic polymers:

Neutral: cellulose, Sephadex, Sepharose.

Polyelectrolyte; CM-cellulose, EMA, aminoethylcellulose.

b) Inorganic carriers: glass, NiO.

The glass is made into an aminoalkylsilane derivative. This is then either converted into the isothiocyanate derivative and linked to the protein by sulfonamide bonds or diazotized and bound to the protein by diazo linkages.

D. IMMOBILIZATION BY INTERMOLECULAR CROSSLINKING

In this method either the protein itself is polymerized and rendered insoluble, or it is bound to a matrix and crosslinked. In most cases bifunctional reagents are used for crosslinking but sometimes inter-molecular bonds are formed between protein molecules.

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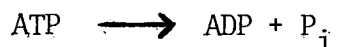
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THE REGENERATION OF ADENOSINE TRIPHOSPHATE

In biological systems, the energy required to drive certain reactions in a direction inherently unfavorable is supplied by so-called "high-energy compounds," of which ATP is the primary example. Hydrolysis of the last pyrophosphate bond of this molecule:



releases approximately 7 Kcal (in vivo this may be as high as 12 Kcal), and this excess energy can be used to drive an appropriately coupled reaction.

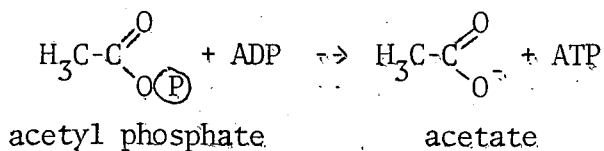
In nature, formation of ATP proceeds by one of the following three mechanisms: 1) substrate level phosphorylation, in which some of the energy released in the course of the degradation of carbohydrates is stored in the high-energy bond of ATP; 2) oxidative phosphorylation taking place in mitochondria, in which oxygen is the terminal electron acceptor, and 3) photophosphorylation, which takes place in chloroplasts and other photosynthetic tissues and in which the energy is supplied by solar light, as explained elsewhere in this report.

The reductive pentose phosphate cycle (Calvin cycle) and the alternate reversed oxidative pentose phosphate cycle and formaldehyde-based cycle proposed in the present report as possible routes for the synthesis of carbohydrates each involve some reactions which use up ATP. The overall energy balance for these processes and the basis of the requirement for ATP are explained in the Introduction. The utilization of ATP and the consequent formation of ADP raise the problem of ATP regeneration, as we wish to have a totally self-sufficient system, in which the only inputs are CO_2 , H_2O , and energy.

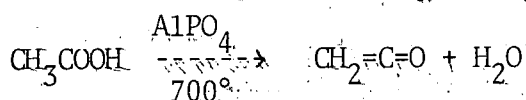
An attempt to reproduce entirely any of the natural ATP regeneration processes appears to us to be futile at this time. Since it is our purpose to synthesize carbohydrates, substrate-level phosphorylation, based on the degradation of sugars, is obviously out of the question. The other processes--oxidative and photosynthetic phosphorylation--involve intricate, membrane-bound electron-transport systems and a series of not completely understood processes in which electrons are moved to successively lower energy states, at the same time using the excess energy for the formation of ATP and the reduction of pyridine nucleotide cofactors. The isolation of these complicated systems in active form and their possible stabilization for long-range industrial use do not appear to be realistic targets for the near future.

We have therefore attempted to delineate other ATP-regenerating procedures which must of necessity involve some chemical reactions not present in the living cell. The procedure outlined below appeared to us quite promising, as all the reactions have already been studied and some are even being used industrially. A plant design based on this series of reactions and the various problems of efficiency, etc., form another chapter of this report.

This method is based on the enzymatic reaction in which ADP is phosphorylated by acetyl phosphate. The enzyme involved is acetate kinase:

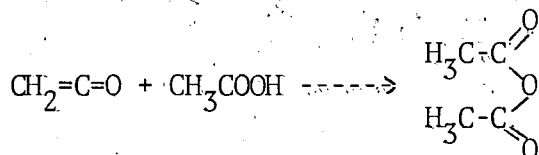


For the formation of acetyl phosphate the following sequence of organic reactions is proposed;

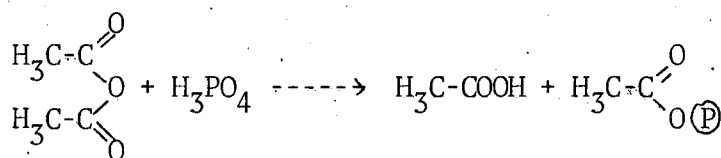


acetic acid

ketene

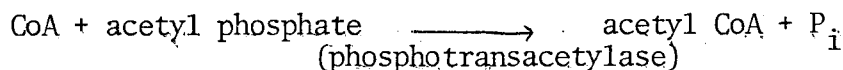
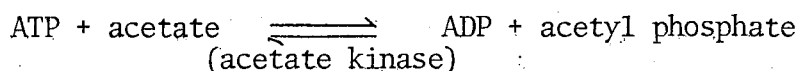


acetic anhydride

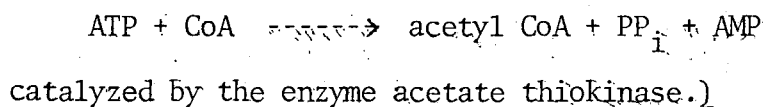


It can be noted that the sequence is cyclic, as acetate and phosphate are reused to make acetyl-P. Of course, since we are making "high-energy" bonds, first in the form of acetyl-P, then as ATP, we must have an input of energy--in this case this is supplied in the form of the heat necessary to dehydrate acetic acid. It is also assumed that acetate can be efficiently recovered from other compounds in the system, so that it can be used for acetyl-P regeneration.

The enzyme, acetokinase (ATP-acetate phosphotransferase, E.C. 2.7.2.1), is present in certain microorganisms where the formation of acetyl coenzyme A, a compound universally required for the metabolic utilization of acetate, proceeds via acetyl phosphate, in the following manner:



(In higher plants and animals the activation of acetate takes place through a different reaction, namely:



The presence of acetate kinase activity has been demonstrated in a number of microorganisms but highly purified preparations have not been reported. The best preparation available to date is that from E. coli (Rose et al., 1954) whose purity was estimated by Anthony and Spector (1970) at 17%. The latter authors also estimated a molecular weight of 46,000. In the direction of ATP formation the kinetic constants were (Rose et al., 1954);

$$K_m \text{ (acetyl phosphate)} = 5 \times 10^{-3} \text{ M}$$

$$K_m \text{ (ADP)} = 1.5 \times 10^{-3} \text{ M}$$

Mg⁺² or Mn⁺² are required by the enzyme:

$$K_m \text{ (Mg}^{+2}\text{)} = 5 \times 10^{-3} \text{ M.}$$

The pH optimum is 7.5 and fairly sharp.

The equilibrium constant at pH 7.3 is greatly in favor of ATP formation:

$$K_{eq} = \frac{[\text{ATP}] [\text{acetate}]}{[\text{ADP}] [\text{acetyl P}]} = 0.006-0.011$$

This means that ΔG of hydrolysis of the anhydride bond of acetyl phosphate is about 3 Kcal higher than that of ATP. In other words, in the proposed ATP regeneration scheme, we are using up this amount of extra energy by going through an intermediate acetyl phosphate stage. In this way the phosphorylation of ADP can be expected to proceed to completion.

The enzyme is quite specific for its substrates. Of other carboxylic acids tested, only propionic acid was active, being phosphorylated ten times slower than acetic acid.

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PART III

Engineering Studies

Charles Wilke

Paul Carroad

Richard Ige

George Mitra

ENGINEERING CONSIDERATIONS

I. Introduction

This section discusses an engineering synthesis of the Calvin cycle of photosynthesis, using insolubilized enzyme reactors. The net reaction which is accomplished is the production of glucose from carbon dioxide and water. In addition to the insolubilized enzyme reactors which carry out the steps of the Calvin cycle, such a system must include regeneration schemes for ATP and NADPH⁺, as well as separation devices to effect the difficult separation of ions which is required.

Figure I-1 illustrates the design configuration to accomplish the reactions. The enzymes of the Calvin cycle are fixed on insoluble matrices in reactors which may be either of fixed bed or of stirred tank design. Water and carbon dioxide enter the sequence, and the carbohydrate product as glucose (or starch) is withdrawn. The schematic diagram is simplified in that it does not show recycle streams or enzyme replenishment.

Table 1 lists the enzymatic reactions which occur in each reactor as numbered. Briefly, ribulose diphosphate and an air stream with carbon dioxide are fed into a stirred tank reactor, in which the enzyme ribulose diphosphate carboxylase may be insolubilized on glass beads. The carbon dioxide is fixed to the ribulose diphosphate and, via an active intermediate compound, 3-phosphoglycerate is produced. The depleted air stream is purged and the PGA is directed to the next enzyme reactor in sequence. The three-carbon compounds are condensed to fructose 6-phosphate, some of which is withdrawn for direct conversion to product and some of which is further reacted within the Calvin cycle to enable continuous operation. Through a series of reactions involving aldolases, transketolases and other

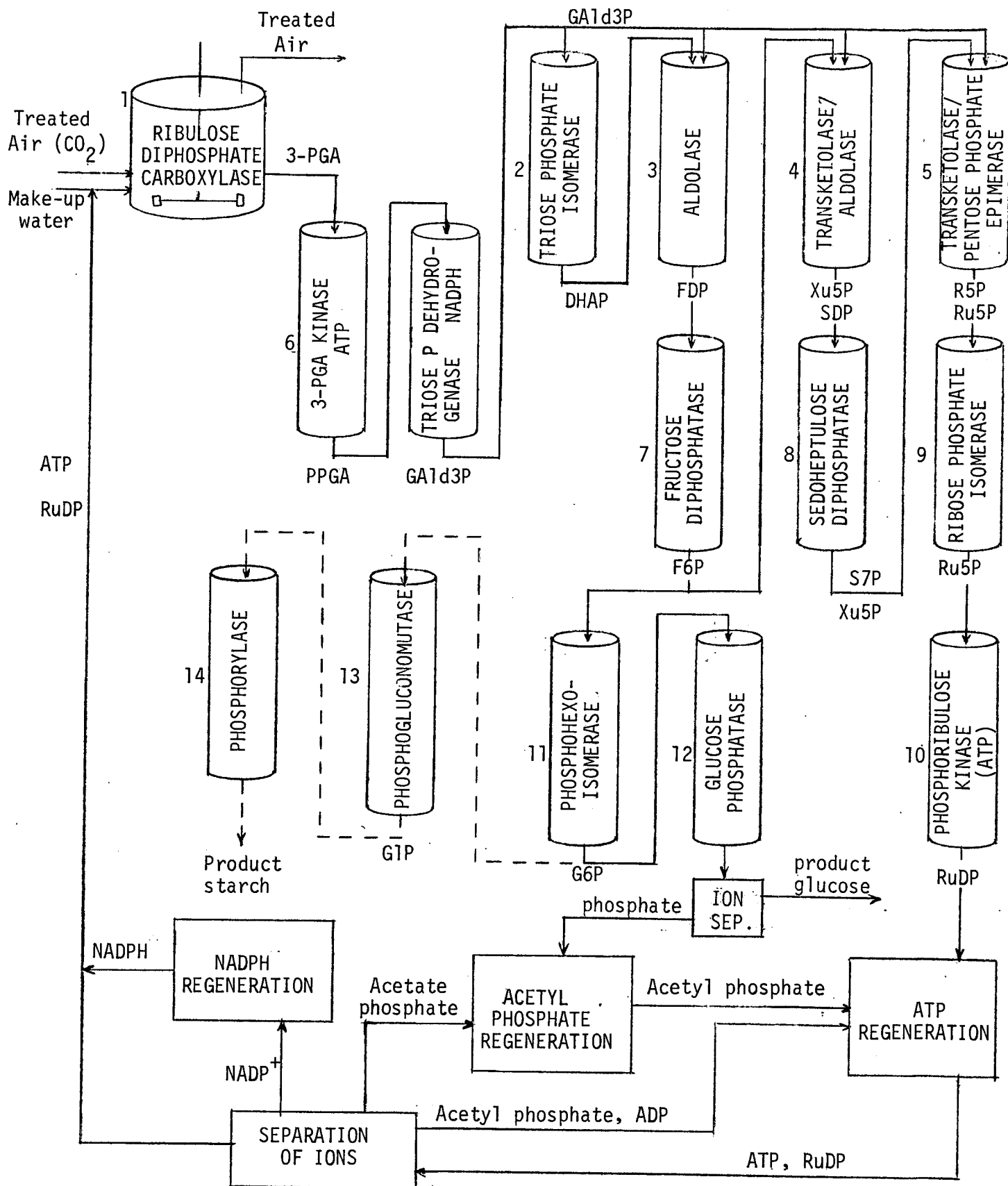


Fig. 1-1 OVERALL PROCESS FLOW DIAGRAM

Table 1

The following reactions occur in Figure 1 as numbered:

1. $\text{CO}_2 + \text{ribulose diphosphate} \xrightarrow[\text{carboxylase}]{\text{ribulose diphosphate}}$ (2)3-phosphoglycerate
2. $\text{Glyceraldehyde 3-P}^2 \xrightarrow{\text{triose phosphate isomerase}}$ dihydroxyacetone phosphate
3. $\text{Dihydroxyacetone phosphate} + \text{glyceraldehyde 3-P}^2 \xrightarrow{\text{aldolase}}$ fructose 1,6-diphosphate
4. $\text{Fructose-6-phosphate} + \text{glyceraldehyde 3-P}^2 \xrightarrow{\text{transketolase}}$ Erythrose 4-phosphate + xyulose-5-phosphate
 $\text{Erythrose-4-phosphate} + \text{dihydroxyacetone-phosphate} \xrightarrow{\text{aldolase}}$ sedoheptulose-1,7-diphosphate
5. $\text{Sedoheptulose-7-phosphate} + \text{glyceraldehyde 3-P}^2 \xrightarrow{\text{transketolase}}$ xylulose-5-phosphate + ribose-5-phosphate
 $\text{Xylulose-5-phosphate} \xrightarrow{\text{pentose phosphate epimerase}}$ ribulose-5-phosphate
6. $\text{3-Phosphoglycerate} \xrightarrow[\text{ATP} \quad \text{NADPH}^+]{\text{3-phosphoglycerate reductase}}$ glyceraldehyde 3-P²
7. $\text{Fructose 1,6-diphosphate} \xrightarrow{\text{fructose diphosphatase}}$ fructose-6-phosphate
8. $\text{Sedoheptulose 1,7-diphosphate} \xrightarrow{\text{sedoheptulose diphosphatase}}$ sedoheptulose-7-phosphate
9. $\text{Ribose-5-phosphate} \xrightarrow{\text{ribose phosphate isomerase}}$ ribulose-5-phosphate
10. $\text{Ribulose-5-phosphate} \xrightarrow[\text{ATP}]{\text{phosphoribulose kinase}}$ ribulose diphosphate
11. $\text{Fructose-6-phosphate} \xrightarrow{\text{phosphohexo isomerase}}$ glucose-6-phosphate
12. $\text{Glucose-6-phosphate} \xrightarrow{\text{glucose phosphatase}}$ glucose α, D
13. $\text{Glucose-6-phosphate} \xrightarrow{\text{phosphogluconomutase}}$ glucose-1-phosphate
14. $\text{Glucose-1-phosphate} \xrightarrow{\text{phosphorylase}}$ starch

specific enzymes, the fructose-6-phosphate regenerates ribulose diphosphate and the cycle repeats. As shown, ATP and NADPH must be regenerated and require special separation procedures.

In Figure I-1, essentially each major enzymatic conversion takes place in a single reactor. Alternatively, a process may be envisioned in which, after the unique reactor to effect the carboxylation which involves a three-phase mixture, as many enzymes as possible are combined within the same stirred tank reactor. It may be possible in this latter case to combine sequential enzymes on the same bead. Figure I-2 diagrams such a scheme. Again the product is taken from the fructose 6-phosphate stream. This mixed enzyme system is the engineering analog to the conception that a green plant cell is a "mixed bag" of enzymes. Clearly, Figures I-1 and I-2 represent only two of the many possible design configurations possible using the Calvin cycle.

In any design, a basis must be chosen which is commensurate with the production level required. For this reason, it is assumed that the gas feed is the effluent from a fossil fuel burning power plant producing 1000 Megawatts of electricity. The plant stack gas stream is assumed to be 2.5×10^6 CFM of which 14% is carbon dioxide. The make-up water is added to the process as needed. Although drawn as a separate entering stream, it may be possible to use the make-up water to advantage by combining it with some operation, such as ion separation.

A consideration of the engineering synthesis of the Calvin cycle of photosynthesis involves many problems. The following sections discuss several of these. Four aspects which are treated in detail are 1) the design of the immobilized carboxylase enzyme reactor using a stirred tank

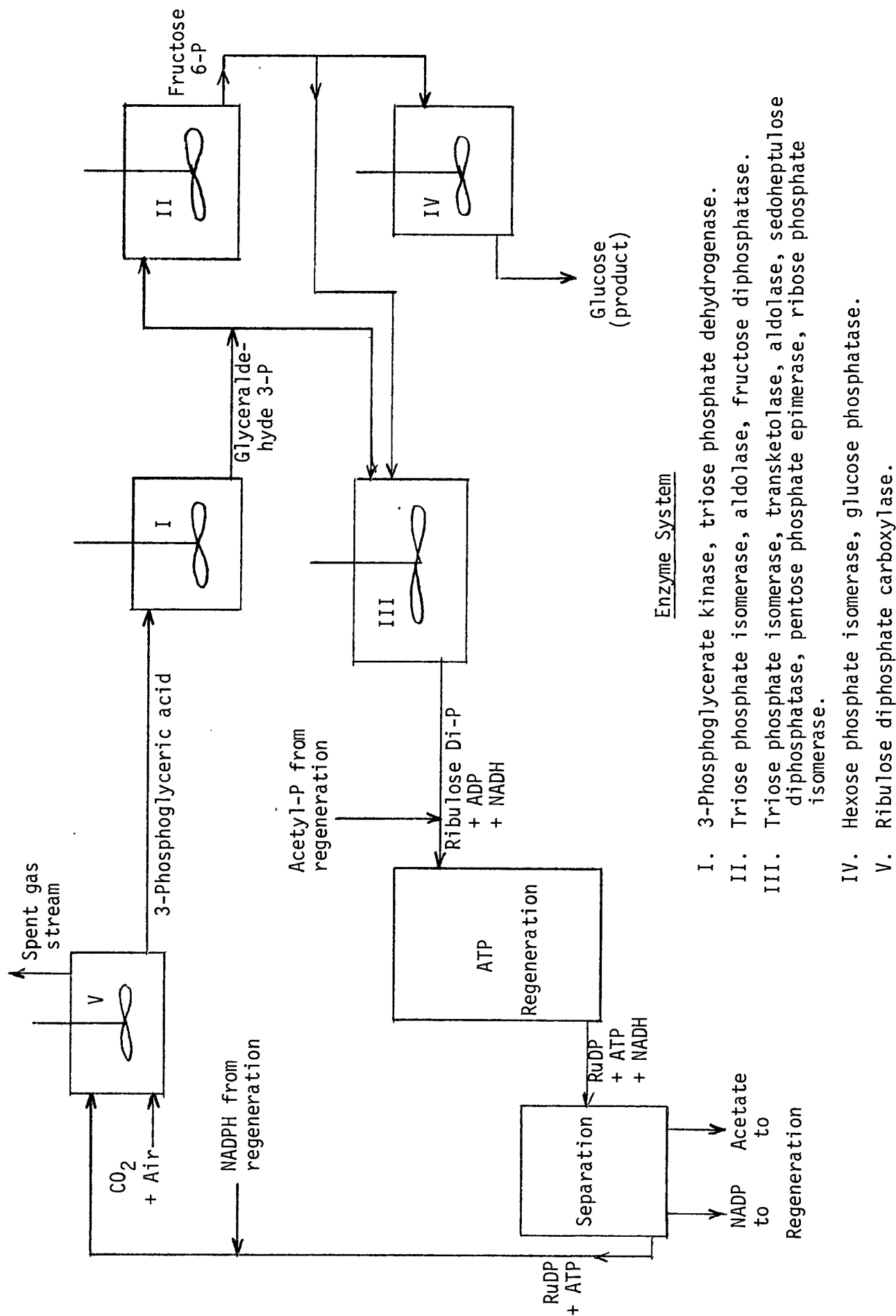


FIGURE 1-2.

design, 2) the design of the ATP regeneration scheme using a packed bed design, 3) consideration of an ion separation system, and 4) energy requirements. Finally, a section is included which discusses some of the engineering problems which have not been explicitly treated, and which may require further development.

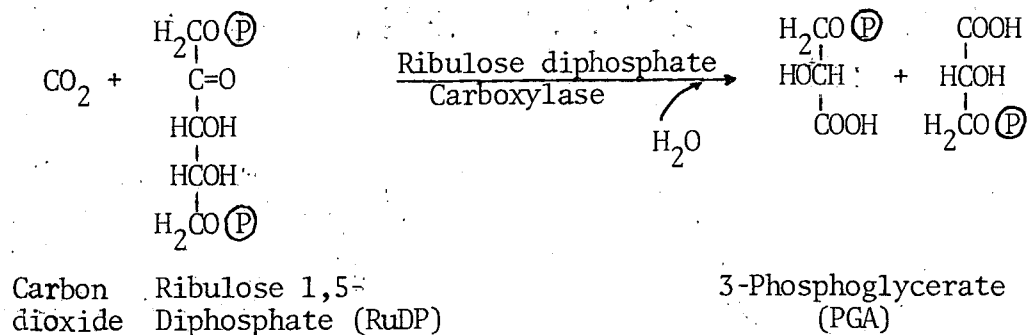
II. Design of a Continuous Feed Stirred Tank

Enzyme Reactor for Production of PGA

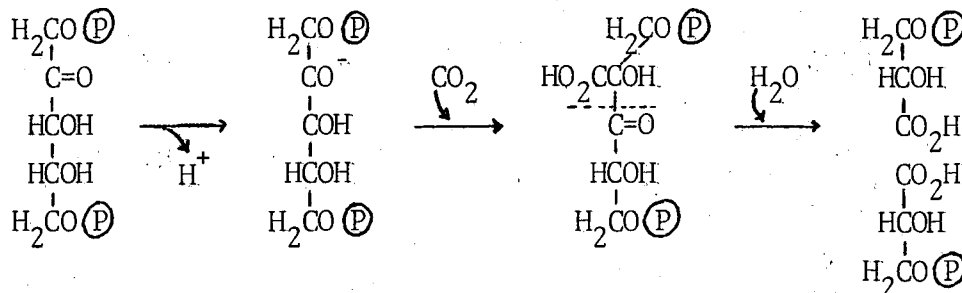
The following describes the design of a continuous feed, stirred tank reactor to accomplish the carboxylation step in the Calvin cycle of photosynthesis on an industrial scale. The method of design is presented. The results are given for a design with porous glass, immobilized enzyme support. Also, a design sketch for a packed tower, an alternative to the stirred tank, is included. The design is not intended to represent an optimization due to time constraints. The many variables are interrelated and it is not possible to solve explicitly for the values to yield the most economic design. Instead, this paper serves to illustrate what assumptions must be made in such a design and to be a guide for more complete studies.

Reaction:

The reaction to be carried out is:



The chemistry of RuDP carboxylation and cleavage to yield two molecules of PGA has been postulated to follow the course shown below: (14)



Design Basis

A design basis must be chosen commensurate with the proposed scale of the carbohydrate production. It is assumed that the carbon dioxide source is the effluent from a fossil fuel burning electrical energy generating plant. A 1000 megawatt electrical energy generating plant emits stack gases of approximately 2.5×10^6 CFM (1) of which 14% is CO_2 (2). From a liquid fuel, one might expect an effluent of 14% CO_2 , 1 - 2 % O_2 , negligible CO, 500 ppm SO_2 for each per cent sulfur in the fuel, and the balance nitrogen (2). Such a gas is assumed to be the plant feed. Scrubbing and cooling operations would be required to remove all components which are harmful to the enzymes and to cool the gases to reaction temperature (30°). Assuming the dissolved CO_2 to be in equilibrium with a CO_2 gas fraction of 3% and assuming conversion of 90% of the dissolved CO_2 , the resulting glucose production is 9.83×10^9 pounds per year.

Enzyme Specifications

1. Equilibrium

The change in free energy for the reaction is -9.8 Kcal per gram mole (13). At 30°C , the equilibrium constant is 10^7 , which is very favorable in the direction of PGA production. It is, therefore, assumed that there is no limitation due to equilibrium.

2. Substrate concentrations

In dealing with enzymes, care must be taken to limit substrate concentrations to levels which are not inhibitory. Dissolved CO_2 in equilibrium with 3% CO_2 in the gas phase, by Henry's Law, is 7.6×10^{-4} molar. For RuDP the maximum substrate level is assumed to be 10^{-3} molar. The effect of running such dilute concentrations of the reactants while demanding high levels of carbohydrate production is to

necessitate very high flow rates.

3. Kinetics

The V_{max} and K_m values are presented elsewhere in this report in the section discussing ribulose diphosphate carboxylase. The assumption is made that the bound enzyme exhibits 10% of the activity of the native enzyme. This reflects activity and K_m variation and any diffusion limitation due to the nature of the porous glass enzyme support. One may separately consider K_m variation (3), but this was not done in this study. It is assumed that the bound enzyme follows Michaelis-Menten kinetics. Since the allowed substrate concentration of RuDP relative to its K_m value is greater than the substrate concentration of CO_2 relative to its K_m value, it is assumed that carbon dioxide is the limiting substrate.

4. Matrix support

In the design, glass beads are chosen as the enzyme support. These are porous, 55 mesh beads. Density of the glass is assumed to be 2.2 gm/cm^3 . The diameter of 55 mesh beads is 0.0182 inches (0.0462 cm). A literature survey of enzyme fixed on porous supports yields an average value of 100×10^{-3} grams of protein per gram of support. For the chosen system, this is 6.25×10^{-3} grams of protein per square meter, if one accepts Weetal's claim that a value of 16 square meters per gram may be achieved with glass of $790 \pm 50 \text{ \AA}$ pore size (4). The possibility of diffusion limitation due to porous support is considered following the design and shown to be relatively unimportant compared to enzyme kinetics.

Two countervailing factors influence the choice of bead size. To minimize diffusion limitations, one decreases bead size. Since for

ribulose diphosphate carboxylase there is little diffusion limitation, the bead size may be increased. Larger beads are easier to screen and hold within the reactor. Smaller beads are easier to hold in suspension, which is essential for good performance. The choice of 55 mesh beads represents a size most commonly reported in the literature.

Design Outline

The many variables involved in such a design interact in such a fashion that direct analytical solution is not possible. A design is refined iteratively subject to certain constraints. Tank geometry was constrained within the limits of standard geometry in the fermentation industry in order to be able to take advantage of several correlations regarding agitation and aeration (6). A tank size was assumed to be the largest which might be available (5). The flow rates must allow the residence time of the substrates in contact with the enzyme for a duration dictated by Michaelis-Menten kinetics. A detailed outline of the design procedure follows. Briefly, however, it might be noted that the design seeks to calculate a minimum liquid residence time (i.e. residence time of substrate in contact with enzyme catalyst) in the reactor subject to conventional design criteria such as gas holdup, liquid entrainment, void volume fraction, etc., and to match this time with that required by the kinetics for a specified conversion. This is an iterative trial-and-error procedure.

1. A value of gas holdup is correlated to superficial gas velocity by Hughmark's correlation (8). Required is density of solution.

Lilly (3) suggests 0.95 for void volume fraction, but Perry's Handbook (11) states that values as low as 0.7 are acceptable. Since the amount of reaction is proportional to enzyme concentration, void volume fraction of 0.8 is chosen.

2. From the assumed size of the reactor (cylindrical tank: 20 feet diameter, 25 feet liquid height, 27 feet total height) the volumes of liquid, holdup, and glass are calculated for void volume fraction 0.8, holdup 15%.
3. The flow rate of gas relates to amount of substrate converted at an assumed conversion (90%). Flow rate per reactor relates to total number of such reactors, given a basis of overall flow rate.
4. From glass specifications and assumed enzyme fixed per unit of glass, the amount of enzyme is calculated. From the volume determinations, the concentration of enzyme is calculated.
5. Concentration of enzyme allows calculation of required residence time for substrate. Since dissolved CO_2 is the limiting substrate, the liquid residence time is crucial. From Levenspiel (7):

$$\bar{\tau} = \frac{V}{F} = \frac{C_{A0}(X)}{-r_A} = \frac{C_{A0}(X)}{\frac{V_{max}C}{K_m+C}}$$

where $\bar{\tau}$ = residence time

V = volume of reactor

F = volumetric flow rate

C_{A0} = entering substrate concentration

r_A = rate of reaction

X = conversion

C = substrate concentration in reactor

6. The necessary liquid flow rate with given concentration of RuDP to stoichiometrically react with the CO_2 at a given conversion can be calculated by mole balance. For a given volume of liquid in the reactor, the residence time of the liquid must match (or at least not be

less than) the required residence time.

7. The residence time of the gas may be calculated.

8. Assuming baffles and a stirrer to be included, correlations (6) are used to estimate the power required for agitation in gassed and ungassed systems. The standard geometry constraint is necessary for use of these correlations.

9. Pressure head of the liquid may be calculated. This represents the pressure over 1 atm to which the gas must be pumped to feed it to the bottom of the reactor. Centrifugal blowers are adequate for the job.

10. Entrainment of liquid by gas must be checked. This must be small for good performance, and this is a variable which "trades off" with high gas throughput. Correlations are presented in the literature (12). At the design conditions, entrainment is very low and not a problem.

11. Heat generation due to the exothermicity of the reaction must be considered. It is reported (16) that 5.0 Kcal are released per gram mole of CO_2 reacted. The design allows for 85.8 gram moles per minute per reactor (0.189 pound moles/min-reactor) of CO_2 converted. Thus, 429 Kcal/min (1700 BTU/min) must be dissipated. This amount of heat exchange may be handled by cooling coils in the tank. Using cooling water, allowing a 15°C rise in the cooling water temperature from for example 10°C to 25°C for a 50°C approach to the reaction temperature of 30°C , would require a flow of 7.56 gallons per minute of cooling water. This is a small effect and may instead be handled by humidification.

12. Diffusion limitations can be a severe hindrance to such a design using a porous catalyst. The carboxylase enzyme, however, is known to

be a particularly sluggish enzyme (14), and it might be expected that the kinetic limitations for this particular case are more severe than pore diffusion. This may be checked by reference to the Thiele modulus, ϕ , and effectiveness factor, η . Satterfield (15) outlines procedures for establishing the importance of diffusion limitation for the Langmuir-Hinshelwood type of rate equation, for which the Michaelis-Menten kinetics is an analog. For the case where either the surface reaction or the adsorption of a single reactant is rate limiting, and allowing for possible inhibition of the reaction rate by either reactants or products, the rate equation is taken to be:

$$\frac{k C_{\text{CO}_2}}{1 + K_{\text{CO}_2} C_{\text{CO}_2} + K_{\text{RuDP}} C_{\text{RuDP}}} = - \frac{1}{V} \frac{dn_{\text{CO}_2}}{dt}$$

where K 's are adsorption coefficients and k is a reaction rate coefficient.

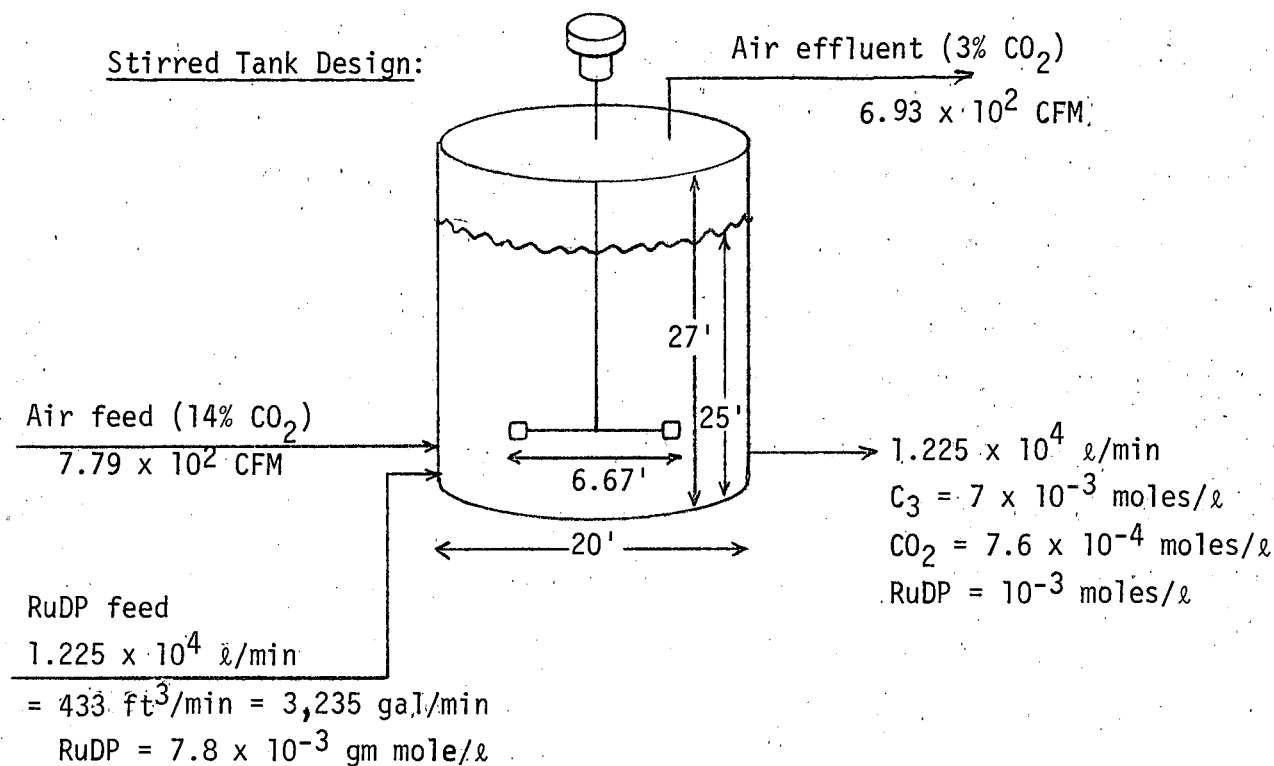
The Thiele modulus for such a situation is:

$$\phi = \left(\frac{L^2}{D_{\text{eff}}} \right) \cdot \left(- \frac{1}{V_c} \frac{dn}{dt} \frac{1}{C_{\text{CO}_2}} \right)$$

where for spherical geometry $L = R/3$ (R = pellet radius). The effective diffusivity, D_{eff} , is assumed to be that of CO_2 in water, $3.57 \times 10^{-5} \text{ cm}^2/\text{sec}$. For the 10% of V_{max} reaction rate assumed to be possible, the Thiele modulus is calculated to be 0.00416. For any $K_A \cdot C_A$ and any reaction order, the effectiveness factor is greater than 0.9. This confirms the expectation that for a very slow enzyme, such as ribulose diphosphate carboxylase, the reaction is kinetically, not diffusion, controlled. This result may also be found as described by Kobayashi (10).

This design does not include the support equipment which such a reactor would require. This includes treatment equipment for the entering gas, temperature and pH control and instrumentation, and various monitoring devices for liquid level, etc. Also, either some sort of screen to hold the glass beads fixed with enzyme in the reactor or a centrifugal separator and recycle system would be necessary. It must also be appreciated that even immobilized enzymes have finite lifetimes and a resupply system should be provided.

A schematic flow sheet and summary of the design calculations are presented on the following page. For a single reactor, a total of 3210 such reactors are required for the specified production.



Specifications
per reactor

ϵ = Void volume fraction = 0.8

Gas holdup = 15%

Density (effective) = 1.24 gm/cm³

Volume of liquid + holdup + glass = 7850 ft³

Volume of dead space = 628 ft³

VVM = 0.099 min⁻¹

Gas velocity = 0.275 feet/sec

Molecular weight of enzyme = 557,000

Enzyme concentration = 7.94 x 10⁻⁵ moles/l

Glass: 55 mesh, porous

Bead diameter = 0.0182 moles = 0.0462 cm

Bead volume = 51.5 x 10⁻⁴ cm³

ρ = density = 2.2 gm/cm³

Assume 100 mgm protein
fixed/gm glass

Pound-moles CO₂ reacted/min = 0.189

Conversion = 90%

Residence time of liquid required
= 12.3 min

Actual residence time of liquid
= 12.4 min

Residence time of gas = 13.65 sec

Power required ungassed = 94.8 HP

Power required gassed = 62.6 HP

Reynolds number = 5 x 10⁵

Power number = 6

Stirrer RPM = 39.1

Pressure feed = 1 atm + .914 atm
= 1.914 atm

Entrainment fraction = $\frac{\text{moles liquid entrained/hr ft}^2}{\text{liquid rate} + \text{moles liquid entrained}}$ < 0.001

Thiele modulus of Satterfield = 0.00416 Efficiency

Thiele modulus of Kobayashi = 0.17 > 0.9

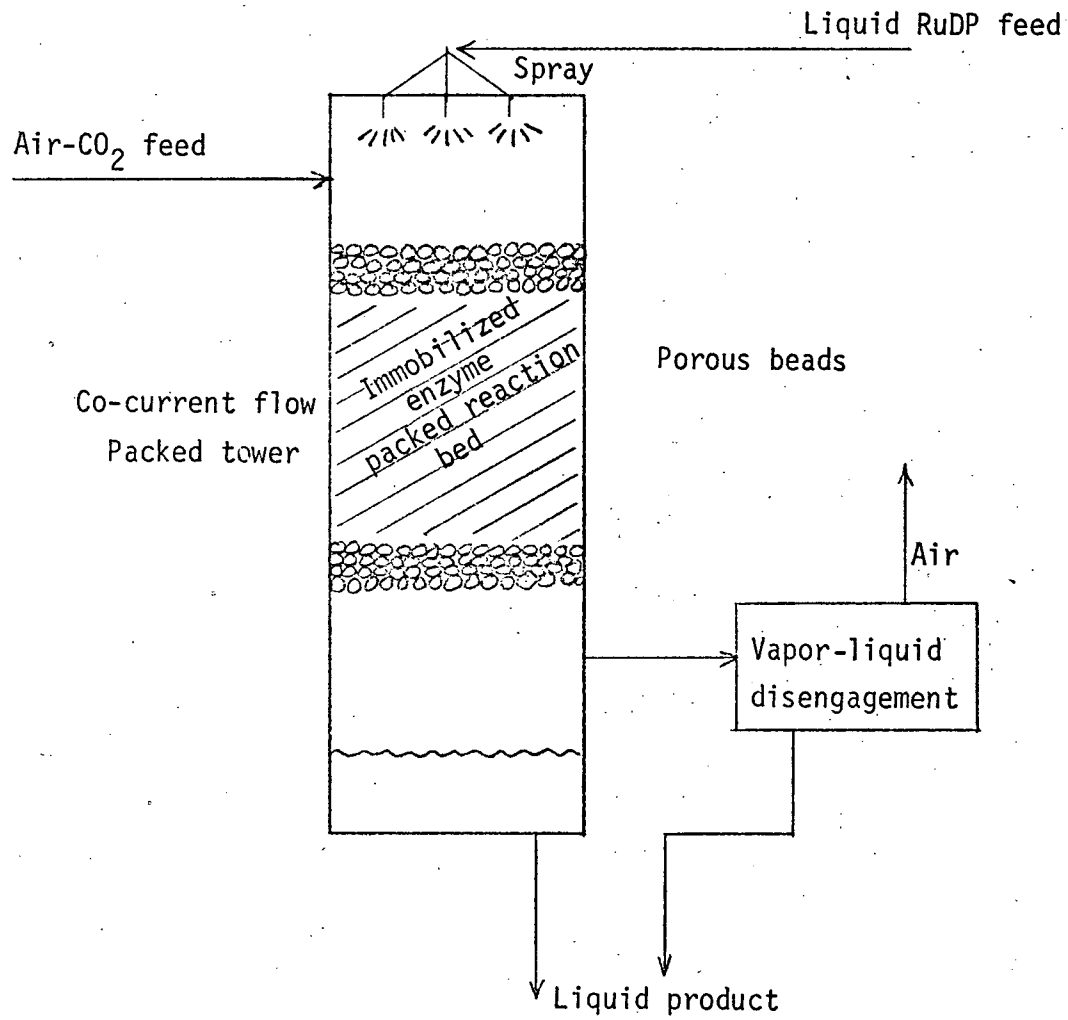
Packed Bed Alternative Design

The following page illustrates an alternative design, a packed bed. Since the enzymatic reaction is slow, the bead size may be significantly increased over 55 mesh, without seriously affecting diffusion control. A large size bead is desirable for packed bed use, as it allows smaller pressure drop through the column.

There is, however, a serious drawback to the packed bed design. The enzyme cannot tolerate a carbon dioxide substrate concentration greater than that in equilibrium with a gas phase fraction of 3% carbon dioxide. In a packed column, then, the maximum substrate concentration is present at the top of the column and the substrate concentration decreases as one progresses down the column. This "less than maximum" concentration of substrate makes for less than maximum utilization of enzyme. Furthermore, the decreasing carbon dioxide concentration makes for a lower concentration gradient for mass transfer. In addition, assuming that the carbon dioxide is available as 14% of the gas effluent from the electrical energy generating plant as before, this stream would have to be diluted to 3% CO_2 before entering the column. In contrast, the stirred tank can operate continuously at maximum substrate concentration.

For details of a packed bed enzyme reactor, reference may be made to the section of this report concerning ATP generation and to reference (10).

Additional Design Proposal:



Conclusion

A design has been presented for a continuous feed stirred tank enzyme reactor. This type of reactor makes efficient use of substrate and enzyme, because it allows the maximum substrate concentration to exist throughout the reactor. Although no optimization has been made on this system, and no quantitative comparison made with a packed bed reactor, this efficient utilization of enzyme and substrate for what is a very slow reaction lends credence to a stirred tank reactor being a judicious choice. Even so, due to the large amount of carbohydrate produced and the low levels of concentration allowed, many such reactors are required.

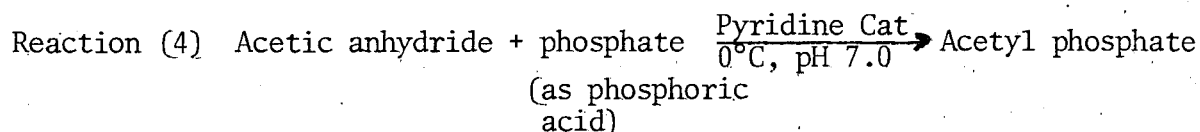
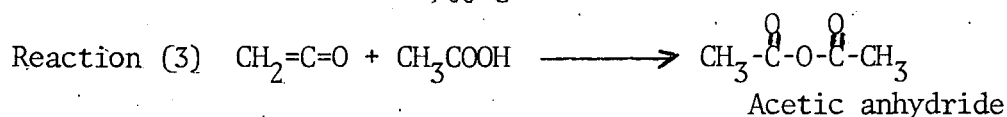
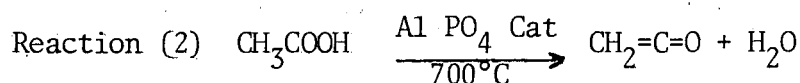
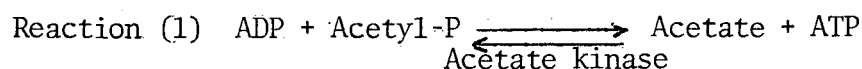
Something ought to be said concerning the magnitude of the numbers presented here. While three thousand reactors may seem an exorbitant number, it must be placed in perspective with the production scale. A production of 9.8×10^9 pounds of glucose per year requires 3.2×10^3 reactors. This scales down to an annual production of several million pounds of product per reactor. This is not unreasonable in conventional terms.

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III. Design of an Enzyme Reactor for ATP Regeneration

Reactions 6 and 10 of the processing sequence outlined in the Introduction (Section I) utilize the high energy phosphate compound, adenosine triphosphate (ATP) as a source of chemical energy. ATP is converted to adenosine diphosphate (ADP) which must be regenerated to ATP in order that the Calvin cycle may proceed continuously. A hypothetical process for ATP regeneration with acetyl phosphate is outlined below, with particular consideration to design of an enzymatic reactor containing acetate kinase bound to glass particles in a packed column. It is assumed that ADP enters the regeneration process following separation from other reactants and products by methods which have not been developed fully in the present study. The proposed reaction sequence is as follows:



The flow sheet of the process is shown in Figure III-1.

In the Calvin cycle 3 moles of ADP are produced per mole of CO_2 converted to glucose. Assuming the same design basis for the PGA reactor described above, the feed stream to the ATP regeneration process will consist of 3.93×10^7 l/m, of aqueous solution with an ADP content of 1727 lb. moles/m. Figure III-1 illustrates the sequence of process steps.

Acetyl phosphate (1727 lb. moles/m) is added and Reaction (1) occurs in the

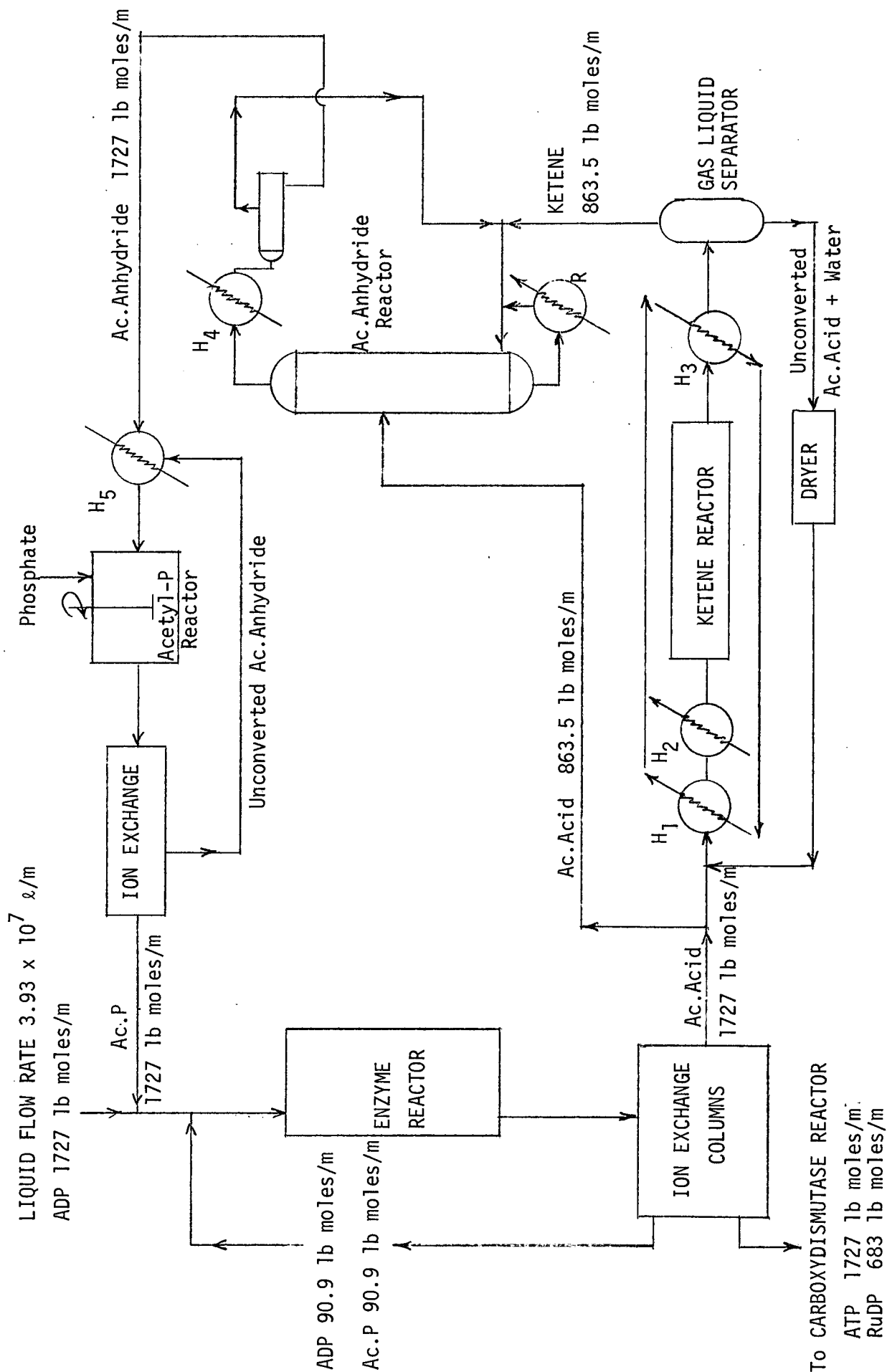


Fig. III-1. FLOW SHEET

enzyme reactor to produce ATP and acetate. A 95% conversion is assumed and the unreacted ADP and acetyl phosphate (90.9 lb mole/m) is recycled. A series of ion-exchange columns is used to separate the effluents of the enzyme reactor. The RuDP (683 lb moles/m) and ATP (1727 lb moles/m) fractions are sent back to the carboxydismutase system at the start of the cycle. The acetate fraction, eluted with HCl to produce acetic acid, is utilized to regenerate acetyl phosphate.

This fraction is split in two equal parts, the first half of which produces ketene (Reaction (2)) in the ketene reactor. Al PO_4 is used as catalyst and conversion per pass at 700°C is about 80% (12). Heat exchangers H_1 and H_3 are coupled (average duty 1.39×10^7 Kcals/min) to heat the incoming stream and cool the effluents respectively. Heat exchanger H_2 is used to control the reactor temperature at the desired level. Reactor products are separated in a gas-liquid separator (b.p. ketene -56°C , b.p. acetic acid 118°C) and the product ketene (gaseous) is sent to the acetic anhydride reactor. The liquid fraction from the separator (mainly unconverted acetic acid and water) is sent to a dryer to remove water and is recycled.

The second fraction of the original acetic acid stream (liquid) is sprayed in the anhydride reactor where it comes in contact with gaseous ketene to produce acetic anhydride (Reaction (3)). The conversion here is fairly high, about 90% (12), and the effluents pass through heat exchanger H_4 which cools it down to 136°C . At this temperature only acetic anhydride is a liquid which is separated from the unreacted gases and transported to the acetyl phosphate reactor.

The present knowledge of Reaction (4) (13) for producing acetyl phosphate from acetic anhydride at 0°C , pH 7.0, with addition of phosphate and

pyridine (catalyst) indicates a low conversion of 40% per pass. Hence, a major separation problem exists with reactor effluents which will require further research. H_5 is a refrigeration system (average duty 5.78 Kcals/m) used to cool the incoming reactants to 0°C.

Description of Process Units

| <u>Item</u> | <u>Description</u> |
|---------------------------|---|
| Enzyme reactor | <p>Packed bed of porous glass beads coupled with acetate kinase enzyme. Reaction taking place</p> $\text{ADP} + \text{acetyl-P} \xrightleftharpoons[\text{Acetate kinase}]{} \text{ATP} + \text{acetate. Temp. } 25^\circ\text{C.}$ |
| Ketene reactor | <p>Reaction.....</p> $\text{CH}_3\text{COOH} \xrightarrow[700^\circ\text{C, AlPO}_4]{\text{Cat}} \text{CH}_2=\text{C}=\text{O} + \text{H}_2\text{O}$ |
| Ac-Anhydride reactor | <p>Reaction $\text{CH}_3\text{COOH} + \text{CH}_2=\text{C}=\text{O}$</p> $\longrightarrow \text{CH}_3-\overset{\overset{\text{O}}{\parallel}}{\text{C}}-\text{O}-\overset{\overset{\text{O}}{\parallel}}{\text{C}}-\text{CH}_3$ |
| Ac-P reactor | <p>Ac-Anhydride + phosphate $\xrightarrow[\text{0}^\circ\text{C, pyridine catalyst}]{\text{pH } 7.0}$ Ac-P</p> |
| H_1, H_2, H_3, H_4, H_5 | <p>Heat exchangers, H_1 and H_3 are coupled and the average duty is 1.39×10^7 Kcals/minute. H_2 is an auxiliary heat exchanger for obtaining the desired temperature of 700°C in the Ketene reactor. H_4 cools the anhydride reactor effluents to 136°C. H_5 is a refrigeration unit, average duty 5.78×10^6 Kcals/minute.</p> |
| R | Reboiler. |
| Dryer | To remove water from the recycled stream of unconverted acetic acid and water. |

Enzyme Reactor Design

1. Theory

Immobilized enzyme reactors have been analyzed by Kobayashi and Moo Young (1) and Lilly, Hornby and Crook (2). The former is a comprehensive analysis taking mass transfer effects in full consideration, together with the enzyme catalyzed reaction given by Michaelis-Menten type kinetics. This is followed in our design.

The reaction is carried out in a packed bed of porous glass beads coated with the enzyme acetate kinase. The properties of the free enzyme have been reported by Rose et al. (3), Rose (4) and most recently by Satchell and White (5). Since all of the studies were made in free solution allowance has to be made for the bound enzyme in our system. Average k_2E (k_2 = M.M. type kinetic const, E enzyme fixed per unit volume of support) is arbitrarily chosen as 1/10th that of free enzyme. This is believed to be a conservative estimate.

Traditional chemical engineering methods are followed in estimation of mass transfer coefficients (6,7) and pressure drop (8) in the bed. It is assumed that there is no previous separation step before the enzyme reactor, and hence the total liquid flow of 3.93×10^7 liters/min, has to be handled.

2. Parameters Used for Design Calculation

$$a) \quad E \equiv \frac{\text{gm of enzyme fixed}}{\text{c.c. of support}}$$

Weetal's (11) work was followed on coupling of trypsin and papain on 96% silica glass (porous, surface area $16 \text{ m}^2/\text{gm}$) and an average value of $5.00 \frac{\text{mg active enzyme}}{\text{gm of glass}}$ was chosen. Proper unit transformation gives

$$E = 1.4 \times 10^{-2} \frac{\text{gm enzyme}}{\text{c.c. glass}}$$

b) $k_2 \equiv \text{Sec}^{-1}$

Satchell and White's (5) reported k_2 in free solution = 1.17 Sec^{-1} . For bound enzyme k_2 was arbitrarily chosen 1/10th of that in free solution. Scanty reports are available in the literature in this matter and experimental work is needed.

c) $K_m \equiv (\text{Michaelis-Menten Const.})$

Value reported by Rose (4) was used, $K_m = 1.5 \times 10^{-3} \frac{\text{moles}}{\text{liter}}$

d) $k_L \equiv \frac{\text{cm}}{\text{Sec.}}$ (mass transfer coefficient in liquid film).

Ranz's (6) correlation was utilized in evaluating k_L after calculating N_{Re} and N_{Sc} , knowing superficial velocity u_s .

e) $\epsilon \equiv \text{void fraction}$

Void fraction was chosen to be 0.4 for the packed tower.

f) $S_o \equiv \frac{\text{gms}}{\text{c.c.}}$

Concentration of ADP in the stream from the carboxydismutase reactor was calculated to be $8.2 \times 10^{-3} \text{ gm/c.c.}$ A 95% conversion was assumed.

3. Integrated Design Equations

The analysis and derivations were obtained from Kobayashi and Moo-Young (1).

For plug-flow conditions the integrated final form of the design equation for the enzyme reactor was

$$S_o(1-Y_e) = (\beta' + 3/4 \alpha') \ln Y_e + \lambda'$$

The symbols are explained under Nomenclature.

4. Results and Discussion

Conversion is assumed at 95%. Table 1(a) compares the performance of porous and non-porous glass beads as enzyme support and the advantage of porous support becomes apparent. Since porous beads possess more surface

area the required height is about 1/2000th of that of a packed bed of non-porous glass beads. However, the enormous pressure drop in either bed rules out the use of a single reactor.

No useful improvement in pressure drop is obtained by decreasing L/D with a single reactor (Table 1(b)). Using 100 reactors, however, the pressure drop is brought down to 3080 H.P.

Table 2 shows calculations for $d_p = 1$ cm. Decrease in L/D ratio from 3.74 to 0.42 decreases pressure drop per reactor from 16,850 H.P. to 55 H.P. A calculation of Thiele parameter, however, rules out use of porous beads with $d_p = 1$ cm, since as shown in Appendix A, this gives an effectiveness factor of 0.094. Because of their large diffusional resistance the glass beads are practically behaving as non-porous supports and the substrate uses only a fraction (~ 0.10) of the available bound enzyme.

Table 3 shows similar results with 1000 reactors and diameter of porous glass beads = 0.1 cm. Pore diffusion consideration for this case (Appendix A) gives a Thiele parameter of 1.0 and an effectiveness factor 0.90. Pressure drop is reduced to a more reasonable level of 27.9 H.P. at L/D = 0.54. A correction factor (corresponding to effectiveness factor of 0.90) may be applied and the length of 0.54 M increased to 0.60 M to take care of pore diffusion.

A practical design for the system might be as follows:

| | |
|---------------------------|-------------|
| Number of enzyme reactors | = 1,000 |
| Length | = 0.60 M |
| Diameter | = 7.94 M |
| Average residence time | = 16.5 Sec. |
| Pressure drop/reactor | = 27.9 H.P. |

The number of reactors is not unreasonably high in view of the large plant capacity of 10^{10} lbs glucose per year and total liquid flow rate of $70,500 \text{ ft}^3/\text{Sec.}$

Table 1

a) Comparison of Porous and Non-porous Supports

Conversion = 95%

| d_p (cm) | Type | E (gm/cc) | No. of reactors | Liquid flow rate (liter/min) | D (M) | L (M) | ΔP^1 (H.P.) |
|---------------|------------|-----------------------|--------------------|------------------------------------|----------|----------|------------------------|
| 1.0 | Porous | 0.014 | 1.0 | 3.93×10^7 | 80.0 | 29.52 | 2×10^6 |
| 1.0 | Non-porous | 1.86×10^{-7} | 1.0 | 3.93×10^7 | 80.0 | 6470.0 | 4×10^8 |

b) Performance Comparison of Single/multiple Reactor with Porous Support

$d_p = 1 \text{ cm; porous}$

$$k_2 E = 1.64 \times 10^{-3} \frac{\text{gm}}{\text{c.c. sec.}}$$

Total liquid flow rate = 3.93×10^7 liters/min.

Conversion = 95%

| No. of Reactors | Liquid flow in each reactor (liter/min) | L (M) | D (M) | Total Reactor Vol. (M^3) | L/D | ΔP (H.P.) |
|--------------------|--|----------|----------|------------------------------------|-------|----------------------|
| 1.0 | 3.93×10^7 | 29.52 | 80.00 | 1.47×10^5 | 0.368 | 2×10^6 |
| 1.0 | 3.93×10^7 | 22.70 | 1130.00 | 2.27×10^5 | 0.002 | 2.93×10^5 |
| 100.0 | 3.93×10^5 | 23.80 | 11.30 | 2.38×10^5 | 2.12 | 3080 |

¹Pressure drop is expressed in term of the equivalent pumping power requirement at the stated flow rate.

Table 2

Effect of Variation in L/D on Reactor Design at $d_p = 1$ cm

Enzyme support: porous glass beads, diameter 1 cm

Liquid flow rate: 3.93×10^7 liters/min.

Number of reactors: 100

Inlet ADP flow rate: 1818 lb moles/min.

Conversion: 95%

$$k_2 E = 1.64 \times 10^{-3} \frac{\text{gms}}{\text{c.c. sec.}}$$

| L (Meters) | D (Meters) | Total Reactor Volume (M ³) | L/D | u (cm/sec) | τ (sec) | ΔP per Reactor (H.P.) |
|---------------|---------------|---|------|---------------|-----------------|-------------------------------------|
| 29.8 | 8.0 | 1490×10^2 | 3.74 | 13.10 | 91.0 | 16,850 |
| 23.8 | 11.3 | 2380×10^2 | 2.12 | 6.55 | 146.0 | 3,080 |
| 10.6 | 25.3 | 5300×10^2 | 0.42 | 1.31 | 323.0 | 55 |

Table 3

Effect of Variation in L/D on Reactor Design at $d_p = 0.1$ cm

Enzyme support: porous glass beads, $d_p = 0.1$ cm

Total liquid flow rate: 3.93×10^7 liters/min.

Number of reactors: 1000

Inlet ADP flow rate: 1818 lb moles/min.

Conversion: 95%

$$k_2 E = 1.65 \times 10^{-3} \frac{\text{gms}}{\text{c.c. sec.}}$$

| L (Meters) | D (Meters) | Total Reactor Volume (M ³) | L/D | u (cm/sec) | τ (sec) | ΔP per Reactor (H.P.) |
|---------------|---------------|---|-------|---------------|-----------------|-------------------------------------|
| 1.92 | 3.56 | 1.92×10^4 | 0.540 | 6.55 | 11.7 | 2470 |
| 0.54 | 7.94 | 2.7×10^4 | 0.068 | 1.31 | 16.5 | 27.9 |

Appendix A

Effect of Pore Diffusion on Design Calculations

The mass transfer resistance inside the pores was neglected during theoretical analysis giving rise to equations used in design calculations. The validity of this assumption will be tested by evaluating the Thiele modulus of the system.

Reaction rate:

$$k_2 \text{ EHS}^* = [k_2 E / (K_m / H)] S^* \quad \text{for } K_m / H \gg S^* \quad (1)$$

$$k_2 E \quad \text{for } K_m / H \ll S^* \quad (2)$$

For present design calculations

$$K_m / H = 3.0 \times 10^{-3} \text{ gm/cc} = 7.0 \times 10^{-6} \frac{\text{gm-mole}}{\text{c.c.}}$$

$$S^* = 2.8 \times 10^{-4} \text{ gm/cc} = 5.6 \times 10^{-7} \frac{\text{gm-mole}}{\text{c.c.}}$$

Therefore: $K_m / H \gg S^*$, and equation (1) applies

This is a first order reaction ($n = 1$) with kinetic constant $k = \frac{k_2 E}{K_m / H}$

$$= \frac{3.24 \times 10^{-8}}{7.00 \times 10^{-6}} = 4.63 \times 10^{-3} \text{ Sec}^{-1}$$

According to Onda et al. (9) the Thiele modulus h for reaction order n is given by

$$h = \sqrt{\frac{n+1}{2} \cdot \frac{k}{D_s} \cdot S^{n-1} \cdot \frac{d_p}{2}} \quad (3)$$

A similar relationship is also given by Petersen (10).

For $d_p = 1 \text{ cm}$

$$h = \sqrt{\frac{4.63 \times 10^{-3}}{10^{-5}}} \times 0.5 = 10.70$$

Effectiveness Factor (Asymptotic Solution for $h > 3.0$) E.F.

$$= \frac{1}{10.70} = 0.094$$

The glass beads are practically behaving as non-porous supports, since the substrate utilizes only a fraction (~ 0.10) of the available surface area bound enzyme. The system is vastly underdesigned.

For $d_p = 0.1 \text{ cm}$

$$h = \sqrt{\frac{4.63 \times 10^{-3}}{10^{-5}}} \times 5 \times 10^{-2} = 1.070$$

$$E.F. > 0.90$$

In this case the internal mass transfer resistance may be neglected since the Thiele parameter is small and effectiveness factor is very near to unity.

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Nomenclature

Any system of consistent units may be used.

a external surface area of support per unit volume of reactor, L^{-1}

D diameter of reactor, L

D_L molecular diffusivity of substrate ADP, L^2/t

D_s diffusivity of substrate within the support (assumed to be $10^{-5} \text{ cm}^2/\text{sec}$), L^2/t

d_p diameter of support particle, L

E enzyme concentration in support, M/L^3

EF effectiveness factor, dimensionless

H partition coefficient, dimensionless

h Thiele modulus

K_m Michaelis-Menten constant, M/L^3

k reaction rate constant when reaction rate is expressed in the form kS^n , $(M/L^3)^{1-n} t^{-1}$

k_L mass transfer coefficient in liquid film, L/t

k_2 rate constant, t^{-1}

L reactor length, L

N_{Re} Reynolds number = $ud_p\rho/\mu$, dimensionless

n order of reaction

r reaction rate, M/tL^3

S substrate (ADP) concentration, M/L^3

S_0 substrate (ADP) concentration of feed, M/L^3

S^* concentration of substrate (ADP) at the surface of support, M/L^3

u superficial velocity of liquid, L/t

Y dimensionless concentration ($=S/S_0$)

Y_e outlet dimensionless concentration of substrate

z dimensionless longitudinal distance = l/L

Greek Letters

α dimensionless parameter = $k_2 E(1 - \epsilon) / k_L a S_0$

$\alpha^* = \alpha S_0, M/L^3$

β dimensionless parameter = $K_m / H S_0$

$\beta^* = \beta S_0, M/L^3$

ϵ void volume per unit volume of reactor, dimensionless

λ dimensionless parameter = $\alpha \omega$

$\lambda^* = \lambda S_0, M/L^3$

ρ density of substrate solution, M/L^3

μ viscosity of substrate solution, M/tL

τ retention time, $L/v, t$

ω dimensionless parameter $(k_L a / \epsilon) \tau$

IV. Separation of RuDP and PGA by Dialysis

An essential consideration in the synthesis of the Calvin cycle is the separation of the products from the various reactors. As an example of the problems involved, a hypothetical process for the separation of PGA from the RuDP-PGA solution coming from the first reactor of Figure I-1 was investigated. The method selected employs a multistage dialysis cascade which is shown schematically in Figure IV-1. A unique feature of the process is the employment of evaporation to establish effective driving forces for diffusion, and also to reduce the membrane area relative to that required for more dilute solutions.

The membranes were assumed to have the properties of the Dow Chemical Co. hollow fiber membranes. These were cylindrical tubes of cellulose with an average pore diameter of 20 Å. From the reported data on the separation of NaCl from raffinose in aqueous solution, and from the previously observed variation of the diffusion coefficients with the solute molar volumes (1) the capacity coefficient for the membranes, U_A , was empirically fitted to a simplified form of the more general equation presented by Perry (2):

$$U_A = 1.02 \times 10^3 V_A^{0.6} (1 + 0.752/F) \quad \text{IV-1}$$

where U_A = overall capacity coefficient for component A, $\ell/\text{cm}^2 \text{ min}$

V_A = molar volume of A, $\text{cm}^3/\text{gm mole}$

F = the Faxen drag factor

The diffusion flux of a given component across the membrane is given by the equation:

$$N_A = U_A (C_{A1} - C_{A2}) \quad \text{IV-2}$$

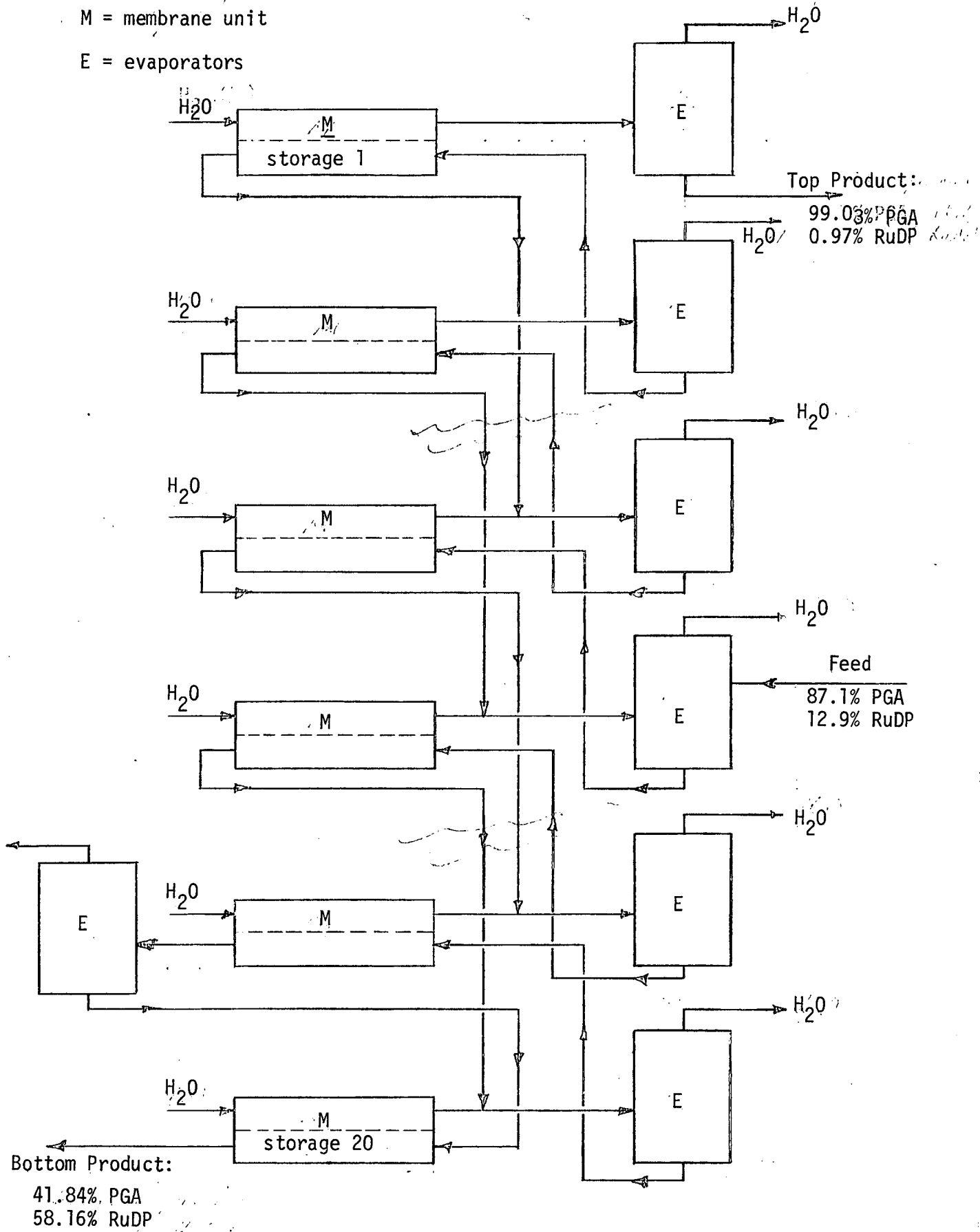
where N_A = diffusion flux of component A, $\text{gm moles}/\text{min cm}^2$

C_{A1}, C_{A2} = concentration of component A on sides 1 and 2, respectively, $\text{gm moles}/\text{liter}$

Fig. IV-1. FLOW DIAGRAM FOR DIALYSIS SEPARATION PROCESS

M = membrane unit

E = evaporators



For any stage, N, in a cascade, the mass balance and flux equations must be satisfied for each component. These equations are:

$$L_{N+1} X_{A,N+1} + V_{N+1} Y_{A,N+1} = L_N X_{A,N} + V_N Y_{A,N} \quad \text{IV-3}$$

$$V_N Y_{A,N} = U_A A(C_{A1} - C_{A2}) \quad \text{IV-4}$$

$$V_N(1 - Y_{A,N}) = U_B A(C_{B1} - C_{B2}) \quad \text{IV-5}$$

where

L = total flow of solute exiting from the feed side of the stage (side 1), gm moles/min

V = total flow of solute exiting from the dialyzate side of the stage (side 2), gm moles/min

X_A, Y_A = mole fraction of component A in the feed and dialyzate streams, respectively

The concentrations on either side of the membrane can be maintained at desired levels by control of the water content of the streams through evaporation and adjustment of the fresh water flow rate into the dialyzate side.

A numerical solution by computer was worked out for the following separation and operating conditions:

Total number of stages: 20

Feed stage (from top): 11

Gross feed rate (before evaporation): 3.93×10^7 g/min

Gross feed composition: $PGA = 6.78 \times 10^{-3}$ M

$RuDP = 1.0 \times 10^{-3}$ M

Net flow to feed stage: 3.93×10^5 g/min

Ratio of solute to solvent: .778 moles solute/liter water

Ratio of dialyzate flow rate to feed flow rate into each stage = 10

Feed composition (water free basis): PGA (mole fraction) = 0.871

$RuDP$ (mole fraction) = 0.129

Top product composition (water free basis): PGA (mole fraction) = 0.9903

RuDP (mole fraction) = 0.0097

Bottom product composition (water free basis):

PGA (mole fraction) = 0.4184

RuDP (mole fraction) = 0.5816

Split of feed (solute only): Top product: 80%

Bottom product: 20%

% Recovery of PGA = 90.96%

Ratio of L to total moles of solute in feed = 1.5

Membrane area required (per mole of feed per min):

| Stage | Area (m ²) | Stage | Area (m ²) |
|---------|------------------------|-----------|------------------------|
| 1 (top) | 7.0239 | 11 (feed) | 25.5978 |
| 2 | 24.2543 | 12 | 25.6859 |
| 3 | 24.3718 | 13 | 25.8324 |
| 4 | 24.4995 | 14 | 26.0755 |
| 5 | 24.6367 | 15 | 26.4754 |
| 6 | 24.7825 | 16 | 27.1213 |
| 7 | 24.9358 | 17 | 28.1318 |
| 8 | 25.0954 | 18 | 29.6361 |
| 9 | 25.2598 | 19 | 31.7190 |
| 10 | 25.4277 | 20 | 34.3352 |

Total area/mole feed/min = 510.5980 m²

Total area for full scale process = 156,140,856 m²
= 1,678,482,732 ft²

Discussion

It appears that a good recovery and purity of PGA is possible with dialysis provided that it is permissible to recycle the bottom product mixture of PGA and RuDP to the first reactor. However, the total cost should be determined for comparison with possible alternative methods.

Detailed analysis of the heat or power requirement for evaporation has not been included in this study. In actual practice heat recovery

would be effected through the use of multi-effect evaporation or vapor recompression principles, in which water vapor from a stage is used to evaporate water in that stage or in other stages at lower pressures.

In the present example, a total evaporation requirement of 4333 lb of water per lb of feed is indicated. By suitable heat recovery arrangements, it might be possible to obtain an evaporation of 10 lb of water per lb of steam supplied for evaporation (or its equivalent in vapor recompression). This would result in a net steam consumption of 385 lb per lb of PGA recovered. This obviously is a serious cost factor amounting to on the order of \$.05/lb of PGA, or \$.10/lb glucose product.

It is not possible to estimate the membrane costs because present prices are based on small scale usage. As a rough estimate, assuming that cost decreases with capacity, the membrane units specified here might cost \$5.00/ft². Approximately 0.337 ft² of membrane is needed per lb of PGA recovered per year. Total membrane cost for the present example would be \$8.4 billion! It should be noted that the present process assumed well-mixed stages, whereas in practice a counter current flow of dialyzate versus feed streams would probably be used. Counter current flow would provide a larger average driving force for diffusion and give a lower membrane area requirement. In this present case, about a 50% reduction in area would result. Therefore, the present design is conservative.

Further study of the present type of dialysis separation will be required to establish optimum arrangements of stages and flow rates. For example, the separation estimated above required 20 stages, whereas the minimum number of stages according to the Fenske equation is 5.2 stages. This suggests that further improvement in the process is possible.

On the basis of the rough costs estimated above, the proposed method does not appear economically feasible at present glucose prices. Very substantial improvements in diffusion rates through the membranes and energy reduction will be necessary. However, the method may be useful in special situations where higher separation expense can be justified.

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V. Energy Considerations

The present process replaces the energy normally received from sunlight in photosynthesis by energy in the form of heat of combustion from fossil fuel or equivalent electrical energy. Major energy inputs are associated with the chemical regeneration of ATP and NADP along with other inputs for separation processes, pumping of fluids, etc.

The minimum energy requirement to produce glucose from CO_2 and H_2O must be the heat of combustion of glucose, or 6730 BTU/lb of glucose. If hydrocarbon oil is burned to produce the CO_2 , i.e., from power plant stack gas, and if it is assumed that only 90% of the CO_2 supplied to the process is converted to glucose because of incomplete absorption in the carboxylase reactor, then 42.7% of the available thermal energy of the power plant fuel would be required to provide the minimum heat for the glucose production. This estimate assumes a normal boiler furnace thermal efficiency of 80%. However, this total energy estimate is conservative because of other inefficiencies of the various process steps.

To provide a more realistic estimate of the energy requirement, the heat necessary to regenerate ADP to ATP according to the process described in Section III was estimated. As a rough estimate, the regeneration energy inputs amount to 42.7 BTU/lb ATP formed. Each lb of ATP releases 24.9 BTU of chemical energy to drive the photosynthetic reactions. The overall thermal efficiency of this step is, therefore, only 58.1%. If it may be assumed that a similar thermal efficiency would be obtained in NADP regeneration, a revised minimum energy input of 11,580 BTU/lb of glucose produced will be necessary. This latter figure is equivalent to 73.4% of the energy available from combustion of the fuel oil.

It would appear, therefore, that the idea of employing this process as a means of utilization of power plant stack gases is probably unrealistic. If other inefficiencies in energy utilization, including separation process requirements are considered there would be very little, if any, energy left for power generation. The combustion facility might as well be designed specifically for carbohydrate production.

It is evident, therefore, that any further engineering assessment of this process should give careful consideration to energy requirements and methods for minimizing all thermal inefficiencies in the various process steps.

VI. Other Engineering Aspects

Fundamental information is needed on the kinetic parameters and yields of the various enzymatic reactions. Suitable enzyme supports and activities in insolubilized form should be determined to permit reactor design for each step.

Further study of energy requirements, including heating and cooling of process streams, pumping and other utility needs is necessary.

Additional consideration should be given to alternative sources of CO_2 of the required purity. Sources of the various enzymes in large quantity must also be developed.

Alternate types of processes should be investigated for separation of inorganic ions in view of the excessive costs estimated for dialysis. Also, the choice of method for separating the product glucose from inorganic impurities must be made.

The foregoing sections have endeavored to indicate a number of representative problems which arise in the assessment of this particular immobilized enzyme process. In addition, there are those aspects which are involved in more conventional chemical process designs.